ABSTRACT

SAGE REIMER DUNLEVY: Stable-Isotope Probing of Anthracene-Degrading Bacteria in a Bioreactor Treating Polycyclic Aromatic Hydrocarbon-Contaminated Soil

(Under the direction of Michael Aitken)

In a prior study examining the degradation of anthracene in untreated soil from a former manufactured-gas plant (MGP), an α-Proteobacterium designated "anthracene group 1" (AG1) was identified as the dominant bacterial anthracene degrader based on recovered 16S rRNA gene sequences during stable-isotope probing (SIP). However, quantitative real-time PCR analysis of AG1 16S rRNA genes of the same soil treated in a lab-scale, slurry-phase bioreactor indicated that these organisms were not likely the primary degraders of anthracene after biological treatment. This study used SIP with [U-^{13}C]anthracene to identify 16S rRNA genes associated with bacteria responsible for anthracene degradation in bioreactor-treated soil. Three bacterial groups were identified as anthracene degraders. One was closely related to members of the genus *Altererythrobacter* and two others were similar to bacteria within the order *Rhizobiales*. Members of the genus *Altererythrobacter* have been previously linked with PAH degradation but usually in a marine environment. *Rhizobiales* have been previously linked with both low- and high-molecular weight PAH degradation. These results suggest that conditions in a bioreactor can enrich for organisms capable of degrading a particular contaminant that are not the same organisms identified as dominant degraders of that contaminant in the untreated soil.

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LIST OF ABBREVIATIONS

EPA	Environmental Protection Agency
HMW	High Molecular Weight
LMW	Low Molecular Weight
MGP	Manufactured-Gas Plant
OTU	Operational Taxonomic Unit
PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
SIP	Stable-Isotope Probing

I. Introduction

The release of polycyclic aromatic hydrocarbons (PAHs) through anthropogenic processes, mainly the incomplete combustion of fossil fuels for transportation and industry, are on the rise. Their recalcitrant properties lead to accumulation in the environment and their potential health effects to humans and animals living in acutely contaminated areas are both numerous and consequential. Through the use of bioremediation, it is possible to lessen both the impact that PAHs have on surrounding environments and their bioavailability, thus lowering their carcinogenic and mutagenic potential. Understanding the process of bioremediation and identifying microorganisms that can degrade PAHs are important steps in the long-term reduction of PAHs in our atmosphere, waterways, and soils. This project helps to broaden the knowledge of how microbial communities adapt to biostimulated conditions to degrade anthracene in PAH-contaminated soils in engineered bioreactor systems.

The objectives of this research project were to identify the bacteria responsible for degrading anthracene in a bioreactor treating PAH-contaminated soil, and to compare those organisms to the microbes identified as dominant anthracene degraders in the untreated soil.

II. Literature Review

This literature review provides information about polycyclic aromatic hydrocarbons including properties, presence in the environment, health effects, bioremediation and degradation, information on anthracene, and prior studies examining PAH-contaminated soil from manufactured-gas plants.

A. Properties of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing fused aromatic rings and are classified into two groups; high molecular weight (HMW) with 4 or more aromatic rings, and low molecular weight (LMW) composed of 3 or fewer aromatic rings (Gnandi et al. 2011; Igwo-Ezikp 2010). Their low water solubility, low vapor pressure, and high octanol-water partition coefficients (K_{ow}), control the transport and distribution of PAHs (International Agency for Research on Cancer 2010) and promote accumulation in the environment (Johnsen et al. 2005). Sixteen PAHs are on the US Environmental Protection Agency (EPA) Priority Pollutant List (Figure 1) (USEPA 1982) due to probable carcinogenic and mutagenic properties (International Agency for Research on Cancer 2010) and their persistent and ubiquitous nature in the environment.

B. Polycyclic Aromatic Hydrocarbons in the Environment

PAHs enter the environment through both natural and anthropogenic processes. Natural sources of PAHs include volcanic eruptions and forest fires. The majority of PAHs in the environment are generated through human activities, specifically incomplete fossil fuel combustion such as from gasoline and diesel engines (Baek 1991; Cavalcante et al. 2012; Nikolaou et al. 1984), residential wood burning (Wang et al. 2010), and industrial activities. Industrial activities include the burning of coal waste piles

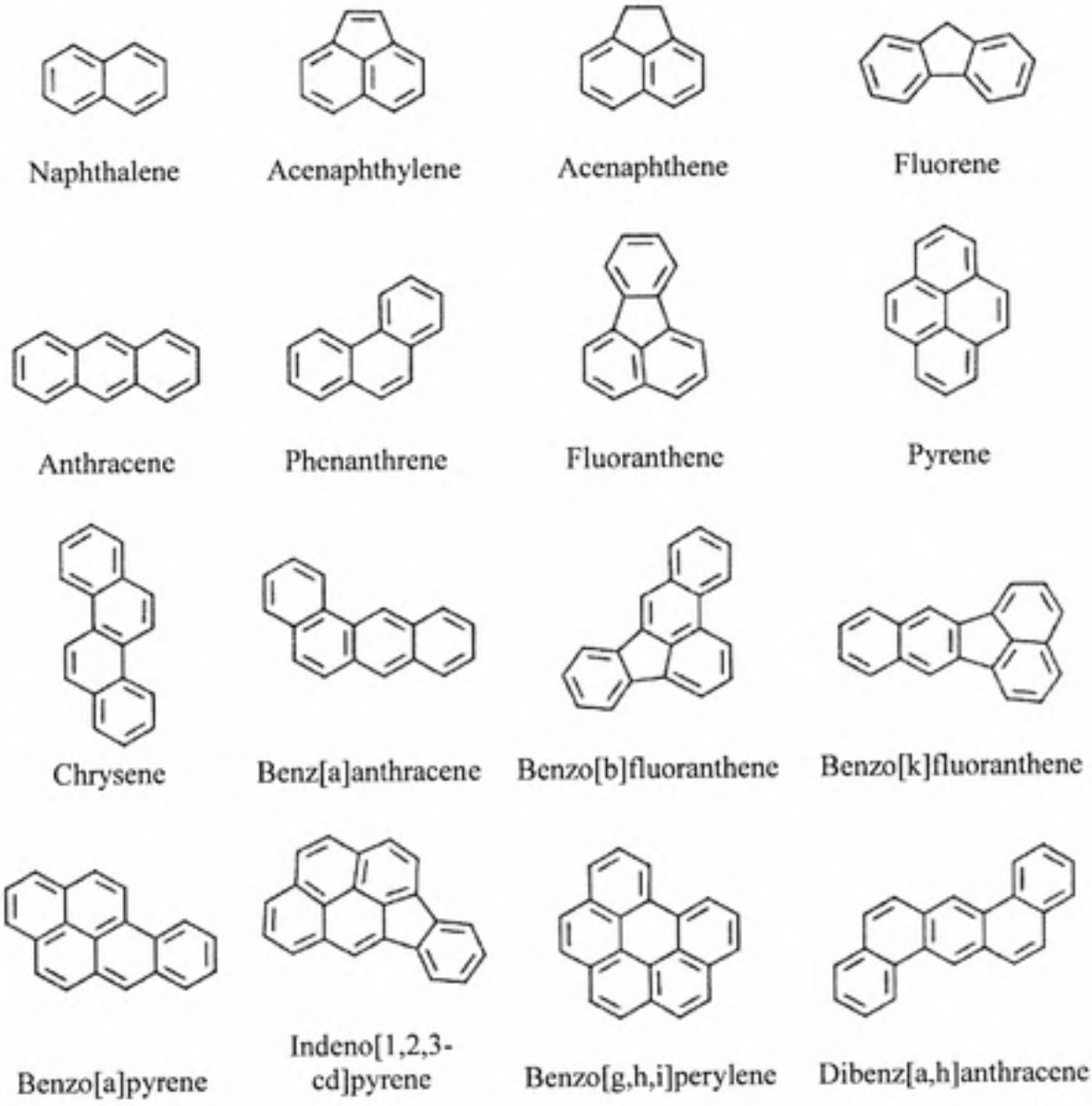


Figure 1: 16 Polycyclic Aromatic Hydrocarbons on EPA's Priority Pollutant List

(Ribeiro et al. 2012), waste incineration (Liu et al. 2010; Park et al. 2009; Wang et al. 2009; Zhao et al. 2008), biomass combustion (Cohn et al. 2011), and disposal of residues from manufactured-gas plants, wood treatment operations that involved creosote, and oil refineries (Bratveit et al. 2007; Eriksson et al. 2000; Otte 1994; Thavamani et al. 2011). Once released into the environment, PAHs can be distributed worldwide through the atmospheric global transport system and contaminate the atmosphere, waters, sediments, and soils.

1. Water and sediment

Waters and sediments of aquatic environments can be contaminated with PAHs through wastewater discharges (Augusto et al. 2011; Qi et al. 2011), runoff of coal tar sealant used on roads and parking lots (Bommarito et al. 2010; Bryer et al. 2010), shipping traffic (Contini et al. 2011), shipping accidents (Guitart et al. 2008; Rogowska et al. 2010), and oil spills (Allan et al. 2012). Rivers, lakes, and estuaries, including the Mississippi River (Brunson et al. 1998; Overton et al. 2004), Potomac River, (Foster and Cui 2008), the Great Lakes (Klecka et al. 2010), and Yangtze estuary (Hui et al. 2009; Li et al. 2012), have all been identified as having detectable levels of PAHs. Due to the hydrophobic nature of PAHs, sediments of waterways are classified as sinks for these compounds and pose health risks for animals living in impacted aquatic environments (Harris et al. 2011; Levengood and Schaeffer 2011).

2. Soil

Soils can become contaminated with PAHs due to fallout from the atmosphere and residuals from prior manufacturing (Thavamani et al. 2011; Thavamani et al. 2011). For example, PAHs have been detected in food sources (Ashraf and Salam 2012) grown

in soils surrounding power plants (Khillare et al. 2012). Ingestion of contaminated food sources is the number one way humans absorb PAHs (Diggs et al. 2011; Menzie 1992). Contamination surrounding manufactured-gas plants (MGP) are of particular interest to industrialized countries because of their prior use and current abandoned state. MGPs are discussed at length below.

C. Health Effects of Polycyclic Aromatic Hydrocarbons

The health effects of PAHs on humans and animals are numerous and have been the subject of increasing studies in the United States and abroad. PAHs are activated by microsomal enzymes resulting in carcinogenic, mutagenic, and toxic metabolites (Levin et al. 1982).

1. Exposure and health effects on humans

Occupational activities including working with oil shale (Kuljukka et al. 1996), oil production (Bratveit et al. 2007; Hopf et al. 2010; Kirkeleit et al. 2006), aluminum smelting (Friesen et al. 2010), and mining (Seidel et al. 2002), release PAHs into the working environment and directly expose specific populations to elevated levels of PAHs. Other occupations including policemen (Topinka et al. 2007) and rickshaw drivers (Rahman et al. 2003), result in secondary exposure to PAHs through atmospheric pollution. Occupational exposure to PAHs has been linked to cardiopulmonary mortality (Friesen et al. 2010), pancreatic cancer (Andreotti and Silverman 2012), and lung cancer (Krick et al. 1993; McClean et al. 2011).

Exposure to PAHs affects men, women, and children differently. Increased exposure in men, for example, manifests in increased infertility rates (Gaspari et al. 2003;

Gu et al. 2010) stemming from sperm DNA adduct formation. PAHs have been shown to negatively impact female fertility (Hombach-Klonisch et al. 2005) and are linked to breast cancer (Mordukhovich et al. 2010; Rundle et al. 2000) and ovarian cancer (Hung et al. 2012). Prenatal PAH exposure has been associated with asthma (Burton 2009), symptoms of attention problems, motor function disorders, mental disorders (Perera et al. 2011), and reduced fetal and child growth rates (Tang et al. 2006).

2. Fish as indicators of PAH contamination

In the environment, fish can serve as natural indicators of PAH contamination because damage to their skin, barbels, fins, and eyes can be observed without specialty equipment (Logan 2007). Industrialized areas record higher rates of DNA damage (Theodorakis et al. 2012; Vincent-Hubert et al. 2012), liver neoplasms (Baumann and Harshbarger 1995; Myers et al. 1990; Varanasi and Stein 1991), body axis defects (Incardona et al. 2005), and immunosuppression (Connelly and Means 2010) in fish and other aquatic animals.

D. Bioremediation

Bioremediation is the process by which microorganisms remove pollutants through metabolism. The overall goal of bioremediation is to lessen toxins' environmental impacts and the threat of exposure. In the case of PAHs, some microorganisms can metabolize these hazardous organic compounds to less harmful products, thereby minimizing potential exposure to PAHs. Due to a number of variables, bioremediation is a site-specific process. The EPA has published a document to help quickly identify the characterization of the site and provided case studies for remediation

processes under multiple conditions (United States Environmental Protection Agency 1999).

1. Bioavailability and toxicity

Bioavailability is the accessibility of a chemical to an organism. In the specific case of PAH and bioremediation, bioavailability refers to accessibility of the chemicals to microorganisms capable of metabolizing those PAHs. PAHs that were recently released into the environment are generally more easily accessible for degradation (Erickson 1993) but PAHs in sediments and soils that have been in the environment for an extended period of time (i.e., "weathered") are less bioavailable (Shuttleworth and Cerniglia 1995) due to the effects of increased partitioning of PAHs into inaccessible domains such as micro-pores and natural organic matter (NOM) (Alexander 2000). Bioavailability is often seen as the limiting factor of bioremediation (Mihelcic 1993) because contaminants are in a different phase than the degrading organisms (Hughes et al. 1997) and increasing the bioavailability of PAHs can help to increase the degradation potential of microbes (Hughes et al. 1997; Volkering 1992).

2. Biostimulation and bioaugmentation

Biostimulation is the addition of nutrients and/or other substances to improve biodegradation by naturally occurring microorganisms, whereas bioaugmentation is the addition of microorganisms with known pollutant transformation capabilities. Biostimulation with oxygen and inorganic nutrients has been shown to remove significantly more PAHs than without (Bamforth and Singleton 2005; Richardson et al. 2012). Bioaugmentation with two different strains of bacteria (*Pseudomonas.spp, bacillus, Pseudomonas aeruginosa*) and a mixture of both strains showed significantly

more degradation of phenantherene-contaminated soil than without bioaugmentation (Nasseri et al. 2010). Not all bioaugmentation practices, however, have proven to be effective, as some isolated organisms fail to degrade once in the field (Megharaj et al. 2011) for multiple reasons, ranging from the possibility that the microorganisms were unable to reach the pollutant, unfavorable survival conditions, and competition with indigenous organisms (Boon and Verstraete 2010). When tested together, biostimulation and bioaugmentation have shown a greater degradation rate than the control in an ex-situ experiment with PAH-contaminated sandy soil from a gas works site (Eriksson et al. 2000).

3. Biotic and abiotic factors

Biotic and abiotic factors must also be taken into account when determining if bioremediation is feasible. These factors include temperature, pH, soil type, aeration, nutrients, depth, diffusion, microbial adaptations, water availability, sediment toxicity, concentrations of the contaminants (Cerniglia 1992), oxygen, microbial population, and chemical structure of the compounds (Haritash and Kaushik 2009).

4. In-situ bioremediation

In-situ bioremediation occurs at the site of contamination when microorganisms use the contaminants for growth. It has been recognized by the National Research Council as cheaper, faster, and safer than conventional cleaning methods should environmental conditions be favorable for such activities (Committee on In Situ Bioremediation, National Research Council 1993). Continuous groundwater flow, minerals that prevent pH fluctuations, and high concentrations of either oxygen, nitrate, sulfate, or ferric iron can produce favorable environmental conditions. Intrinsic

bioremediation, or natural attenuation, and engineered bioremediation can be used in in-situ applications.

5. Ex-situ bioremediation

Ex-situ bioremediation involves removing the contaminated soil and treating it under controlled, ideal degradation conditions. For example, bioreactors can be used to increase the mass-transfer rate by increasing the total surface area through agitation (Deziel et al. 1999). Several studies have demonstrated the efficiency of bioreactors for decreasing PAH concentrations in contaminated soils (Launen et al. 2002; Lundstedt et al. 2003; Singleton et al. 2005; Villemur 2000). Composting has also been used as a form of bioremediation for PAH-contaminated soils, although with limited effect on the overall toxicity of the soils (Cajthaml et al. 2002; Reichenberg et al. 2010).

E. Degradation of Polycyclic Aromatic Hydrocarbons

There is a stark distinction between the degradation of HMW PAHs and LMW PAHs. HMW PAHs tend to be more recalcitrant to microbial attack due to molecular stability (Providenti 1993) and lower solubility, whereas LMW PAH degradation occurs more easily and has been extensively studied (Lundstedt et al. 2003; Samanta et al. 2002), with numerous degraders identified. Of the HMW PAHs, pyrene has been the best-studied with respect to isolation of bacterial degraders and elucidation of metabolic pathways.

1. Pathways associated with PAH utilization/degradation

Bacterial isolates, bacterial consortia, fungi, algae, and a bacterial-fungal complex have all demonstrated an ability to degrade both HMW and LMW PAHs through a

variety of pathways (Boonchan et al. 2000; Cerniglia 1992; Li et al. 2008; Yuan 2000), as shown in Figure 2.

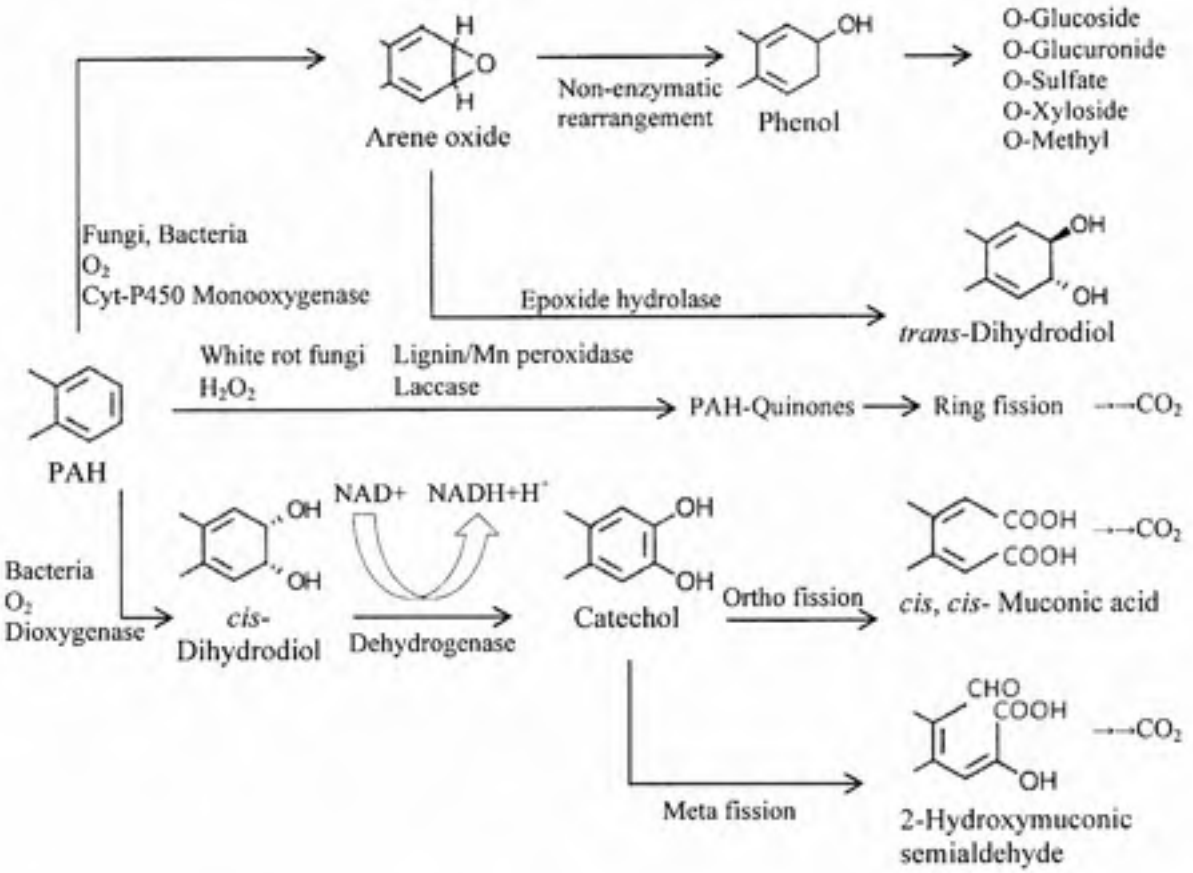


Figure 2: Proposed Pathways for Microbial Catabolism of Polycyclic Aromatic Hydrocarbons (Cerniglia 1992)

Microbial degradation of PAHs occurs in one of two ways, either as the sole source of carbon and energy to the microorganism or by co-metabolism (or co-oxidation if oxidation occurs) (Wilson and Jones 1993). Co-metabolism, despite not directly benefiting the microorganism, is extremely important in the overall degradation of PAHs because it can pave the way for additional degradation by another microorganism. Most often, bacteria incorporate both atoms of molecular oxygen by utilizing dioxygenase

enzymes to form an intermediate *cis*-dihydrodiol. The *cis*-dihydrodiol is dehydrogenated by a *cis*-dihydrodiol dehydrogenase to form a dihydroxylated intermediate. This intermediate is further oxidized to form a ring-cleavage product through an *ortho* or *meta* cleavage pathway (Cerniglia 1992; Johnsen et al. 2005; Kanaly 2000; Vandermeer 1992; Zeyaullah 2009).

Fungi utilize cytochrome P450, a monooxygenase enzyme, by incorporating one atom of molecular oxygen into the aromatic nucleus, resulting in an unstable arene oxide intermediate. The arene oxide intermediate undergoes further metabolism by epoxide hydrolase to form a *trans*-dihydrodiol or it can be nonenzymatically rearranged to form phenol (Cerniglia 1992; Haritash and Kaushik 2009; Sutherland 1992).

2. Microorganisms associated with PAH utilization/degradation

Many microorganisms have been studied for their PAH degradation properties and a diverse community has been identified (Lu et al. 2011). PAH degradation has been identified in aerobic conditions, nitrate-reducing conditions, sulfate-reducing conditions (Lu et al. 2011), and other anaerobic conditions (Meckenstock and Mouttaki 2011). The most-studied PAH-degrading bacteria are from the genera *Pseudomonas*, *Sphingomonas*, and *Mycobacterium*.

F. Anthracene

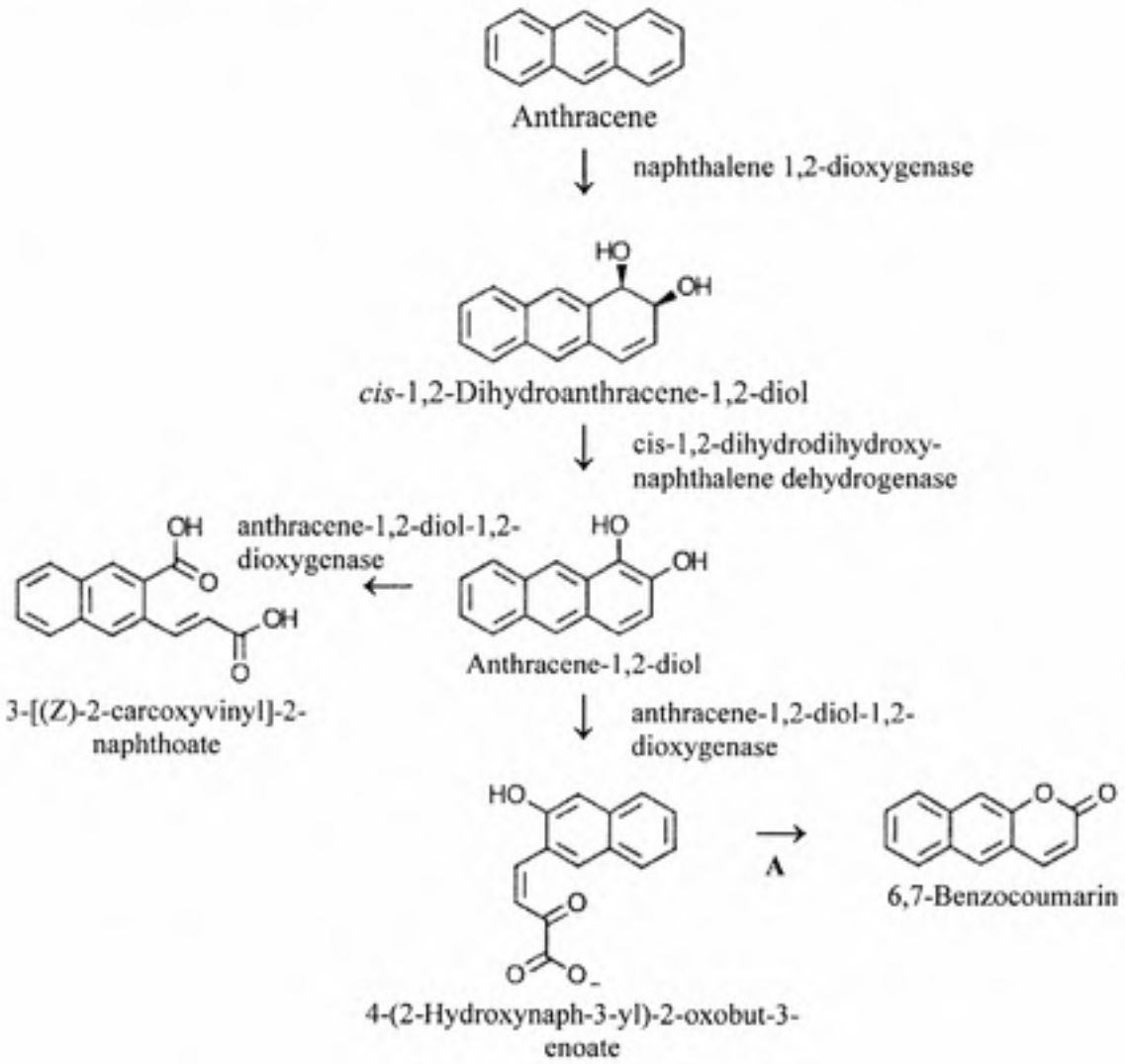
1. Chemical and physical properties

The chemical structure of anthracene (Figure 1) comprises three benzene rings in a linear arrangement. It has a chemical formula of $C_{14}H_{10}$, and a molecular weight of 178.24 daltons. As a pure compound, anthracene is a white powdery crystal. Its boiling

point is 340°C and melting point is 218°C. Like other PAHs, anthracene is highly hydrophobic with an aqueous solubility of 0.13 mg/L (International Programme in Chemical Safety 1999) although it will dissolve in strong organic solvents. The legal airborne permissible exposure limit is 0.2 mg/m^3 averaged over an 8-hour work shift (United States Department of Labor).

2. Proposed degradation pathway

The proposed pathway for bacterial metabolism of anthracene (Geldner et al. 2011) is shown in Figure 3.



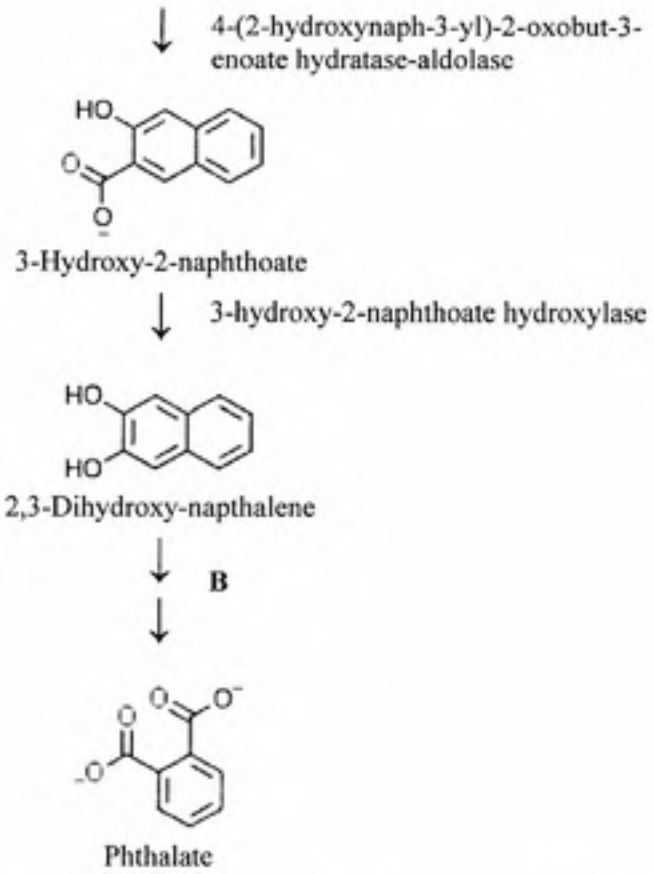


Figure 3: Proposed Pathway for Anthracene Degradation (Geldner et al. 2011)

In the first step of anthracene degradation (Geldner et al. 2011), anthracene undergoes hydroxylation of the aromatic ring, resulting in *cis*-1,2-dihydroanthracene-1,2-diol. This intermediate is converted to anthracene-1,2-diol, which is cleaved at the meta position resulting in 4-(2-hydroxynaph-3-yl)-2-oxobut-3-enoate or it can be cleaved at the ortho position and results in 3-[(Z)-2-carboxyvinyl]-2-napthoate. 4-(2-Hydroxynaph-3-yl)-2-oxobut-3-enoate may spontaneously rearrange to form 6,7-benzocoumarin or be converted to 3-hydroxy-2-naphthoate. 3-Hydroxy-2-naphthoate undergoes transformation by means of a hydroxylase, resulting in 2,3-dihydroxynaphthalene, which is converted into phthalate through an unknown mechanism.

3. Previous studies identifying anthracene degraders

Anthracene has not been extensively examined as the sole PAH in biodegradation studies. This does not mean, however, that microorganisms that are capable of anthracene degradation are left unstudied or unexamined. Jones et al. (2011a) examined anthracene degradation utilizing stable-isotope probing in an aerobic environment and identified uncharacterized anthracene-degrading bacteria called 'anthracene group 1" in the *Sphingomonadales* order. Zhang et al. (2011) identified three microorganisms not previously linked to anthracene degradation under methanogenic conditions that were classified within the *Methylibium, Legionella,* and *Rhizobiales* genera. Dean-Ross et al. (2001) examined the degradation of anthracene by a *Rhodococcus* species observing that it was able to utilize anthracene, phenanthrene, pyrene, and fluoranthene. Pathak et al. (2008) identified an isolated *Pseudomonas* strain as capable of degrading anthracene in addition to phenanthrene. Van Herwijnen et al. (2003) isolated *Mycobacterium* sp. strain LB501T and identified it as an anthracene degrader. The fungus *Apsergillus fumigatus* has also been shown to degrade anthracene (Ye et al. 2011).

4. Concerns about anthracene from the international community

Unlike some PAHs, anthracene is not considered carcinogenic to humans. It is however, on the EPA's Priority Pollutant List (USEPA 1982), noted on the Substances of Very High Concern list by the European Chemicals Agency (European Chemicals Agency 2008), and identified as toxic to aquatic organisms and environments on the International Chemical Safety Card issued by the International Programme in Chemical Safety (International Programme in Chemical Safety 1999).

G. Manufactured-Gas Plants

1. Historical information

Manufactured gas was commercially developed in England between 1795 and 1805 and was used for lighting, heating, and cooking purposes. Manufactured-gas plants (MGP) were centrally located in towns to decrease delivery time and distribution length. The first MGP in the United States was operational and servicing Baltimore, Maryland in 1816 (Harkins et al. 1988). Byproducts of the manufacturing process were complex mixtures such as tars containing PAHs, which were subsequently stored in pits or in tanks below ground level around the plants. Coal tar has an adhesive-like texture and contains a complex mixture of PAHs and other toxic molecules. Manufacturing of gas ceased and plants were closed in the 1960s due to increased production of piped natural gas and other energy products. Due to a lack of environmental regulations and unknown health effects, the centrally located coal tar pits or tanks of approximately 3,000-5,000 MGPs throughout the United States were simply covered up or left exposed to the elements when the plants were closed (United States Environmental Protection Agency 1999). Due to higher solubility of LMW PAHs in groundwater and the fact that LMW PAHs are more susceptible to volatilization and degradation, the more difficult to-degrade carcinogenic and mutagenic HMW PAHs are often in higher concentrations at these sites.

The MGP that was the source of the soil in this study was located in Salisbury, NC. Figure 4 shows the exact location of the plant.

Figure 4: Location of Salisbury Manufactured-Gas Plant

2. Prior case studies of PAH degradation at MGP sites

Manufactured-gas plants were relevant for much of the industrialized world for several decades and residual contaminants from those sites are therefore a worldwide problem. Several studies have examined microorganisms or processes that contribute to the bioremediation of PAHs at these MGPs.

In the early 1990s, Erickson et al. (1993) examined the feasibility of PAH bioremediation of MGP soils using small batch microcosms and found that only spiked PAHs were being degraded while the indigenous PAH concentrations remained unchanged throughout the course of the experiment, thus concluding that the PAHs in the soils were unavailable for microbial degradation. He found this to be true when he spiked the soil with known PAH degraders as well.

Lundstedt et al. (2003), examined not only the degradation of PAHs during bioslurry treatment of soils from an MGP site, but also examined the formation of oxy-PAHs throughout the study. The LMW PAHs were found to degrade faster than the HMW PAHs. The formation of PAH metabolites during degradation was monitored and it was discovered that oxy-PAHs, which can be acutely toxic, mutagenic, and carcinogenic, were possibly being formed as a by-product of PAH degradation. A subsequent study (Lundstedt et al. 2007) outlined the sources, fate, and additional hazards of oxy-PAHs formed during the biodegradation process.

Boldrin et al. (1993), were able to isolate from an MGP site, the bacterium *Mycobacterium* sp. strain BB1 which was capable of degrading phenanthrene, fluoranthene, and pyrene. They additionally illustrated that a co-metabolic process was employed by *Mycobacterium* sp. strain BB1 enabling it to successfully degrade fluorene.

Other investigators (Eriksson et al. 2000; Grosser et al. 1991; Otte 1994) have concluded that inocula of PAH-degrading bacteria originally derived from PAH-contaminated soils were able to increase the efficiency of and lessen the time required for PAH degradation.

H. Stable-Isotope Probing

The use of stable-isotope probing (SIP) in bioremediation studies helps to identify metabolic functions of microorganisms without isolation (Radajewski et al. 2003). SIP works by providing microorganisms with a stable-isotope labeled-compound as a source for growth. Once integrated into the microorganism's cellular structure, the labeled atom can be used to identify the microorganisms that metabolized the substrate. For example,

when a uniformly ^{13}C-labeled organic compound is used for growth, the ^{13}C atoms are incorporated into new cellular macromolecules, including DNA. The more-dense ^{13}C-enriched DNA can then be physically separated from unlabeled DNA by ultracentrifugation. In this study, 16S rRNA genes were examined for incorporation of the ^{13}C from [U-^{13}C]anthracene.

Traditionally, ethidium bromide (EtBr) has been used as a means to visualize the separation of the isotope-labeled nucleic acids and the non-isotope-labeled nucleic acids. Alternative techniques have been developed, including the elimination of EtBr which can be damaging to the DNA in the SIP experiment and must be disposed of in a safe manner. The use of SYBR safe (Martineau et al. 2008) is now considered a viable alternative.

This cultivation-independent tool has become important in helping to identify microorganisms responsible for the degradation of PAHs in complex mixtures under a variety of environmental conditions. For example, SIP has been used to identify bacteria capable of degrading naphthalene (Jeon et al. 2004; Singleton et al. 2005), anthracene (Jones et al. 2011a; Zhang et al. 2011), phenanthrene (Singleton et al. 2005; Singleton et al. 2007), pyrene (Jones et al. 2008; Singleton et al. 2006), and fluoranthene and benz[a]anthracene (Jones et al. 2011b). In addition, Gutierrez et al. (2011) were able to identify LWM PAH degraders in an algal bloom. While useful for identifying uncultivated microorganisms, SIP has also assisted in the isolation of identified PAH degraders (Jeon et al. 2004; Singleton et al. 2009).

This lab has previously used SIP and PAH-contaminated soil from the Salisbury, NC site to show for the first time that *Pigmentiphaga*-related sequences were responsible

for the degradation of naphthalene and phenanthrene and that a group of microorganisms known as 'pyrene group 2' were associated with degradation of pyrene, fluoranthere and benz[*a*]anthracene (Jones et al. 2011b). In another SIP study from this lab, an anthracene degrader was identified with no genetically close, characterized relative (Jones et al. 2011a). The sequences associated with anthracene degradation were therefore referred to as 'anthracene group 1' (AG1). All previous SIP studies on the Salisbury soil were conducted on untreated soil.

III. Materials and Methods

A. Chemicals and Soil

All chemicals and solvents were molecular biology grade or high-pressure liquid chromatography (HPLC) grade except for unlabeled (^{12}C) anthracene, which was scintillation grade. [1,2,3,4,4a,9a-^{14}C]anthracene was obtained from Sigma-Aldrich (St. Louis, MO). PAH-contaminated soil was obtained from Salisbury, Rowan County, North Carolina, from the site of a former manufactured-gas plant and processed as previously described in Jones et al. 2011b. [U-^{13}C]anthracene was synthesized as described elsewhere (Zhang et al. 2011).

B. Preliminary Experiments

1. Attempt to isolate "anthracene group 1"

One gram wet weight untreated soil was mixed with 15 mg unlabeled anthracene and 30 mL simulated groundwater amended with inorganic nitrogen and phosphorus containing 0.37 mM NH_4NO_3, 0.08 mM K_2HPO_4, 0.7 mM $CaCl_2 \cdot 2H_2O$, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM $NaHCO_3$, 0.06 mM KCl, and 1 N H_2SO_4 and incubated on a rotary shaker at 225 rpm and 25°C for 5 days. Samples from that enrichment were serially diluted onto plates of modified 216L agar (containing per liter of simulated groundwater, 1 g sodium acetate; 10 g tryptone; 2 g yeast extract; 0.5 g sodium citrate; 1.5% agar; pH 7.5) and R2A agar (Noegen, Lansing, MI). Plates were incubated in the dark at 37°C for 3-7 days and were removed once ample colony formation was visible. Isolated colonies were screened for the presence of "anthracene group 1" using whole-cell template PCR and specific primers developed previously (Jones et al. 2011a). The

PCR program for those primers consisted of 15 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 57.5°C, and 30 seconds at 72°C, and ended with a final dwell at 72°C for 3 minutes. Products were examined using a 2% agarose gel. As a positive control, a plasmid clone containing a 16S rRNA gene for a member of AG1 from the previous SIP experiment with anthracene was used.

2. "Anthracene group 1" and pH measurements in bioreactor-treated soil

To quantify the relative abundance of genes associated with AG1 in biostimulated, untreated soil as well as in bioreactor-treated soil, an experiment was conducted measuring the change in total bacterial 16S rRNA genes and AG1-specific 16S rRNA genes over a 114 h time period. One gram dry weight of either untreated soil or bioreactor-treated soil was added in triplicate to 30 mL of either simulated groundwater or reactor buffer (5 mM phosphate, 5 mM NH_4NO_3) with 12.5 mg anthracene. The abundance of total bacterial and AG1-specific 16S rRNA genes was determined using quantitative real-time PCR (qPCR) with primers specific for each of those groups (Jones et al. 2011a) on the untreated soil with simulated groundwater at 0 h, and the bioreactor-treated soil with simulated groundwater at 0 h. qPCR was also performed on each sample incubated in either simulated groundwater or reactor buffer at 114 h. The pH of each sample was monitored (Expandable ionAnalyzer EA920; Orion Research, Cambridge, MA) at time points 0 h, 20 h, 42 h, 95 h and 114 h.

3. Effect of incubation medium on pH of the bioreacted-treated soil

To test if nitrification of ammonium in the medium used in incubations with the bioreactor-treated soil affected the pH of the incubations (as described in the previous section), 1 g dry weight of bioreactor-treated soil was washed with either simulated groundwater or reactor buffer five times by centrifugation at 3,500 rpm for 7.5 minutes each and the supernatant decanted each time. The washed soil was incubated as described above, and the pH was measured at 0 h and 121 h. Incubations were conducted in triplicate.

4. Effects of ammonium nitrate concentration on slurry pH, mineralization, and anthracene disappearance

To determine the effect of the ammonium nitrate concentration in amended phosphate buffer on pH over time, an experiment on the bioreactor-treated soil was conducted in which the concentration of phosphate in the reactor buffer was maintained at 5 mM and the ammonium nitrate concentration was reduced to either 2.5 mM or 1.0 mM. The pH of the incubations containing 2.5 mM ammonium nitrate was measured at 0 h, 29 h, 49 h, 65 h, 96 h, 117 h, and 306 h. The pH of the incubations containing 1.0 mM ammonium nitrate was measured at 0 h, 24 h, 48 h, 69 h, 95 h, 117 h, and 456 h.

The effect of different ammonium nitrate concentrations was tested on the mineralization of ^{14}C-anthracene. Additionally, the disappearance of anthracene in both untreated soil and bioreactor-treated soil was determined using unlabeled anthracene. For untreated soil, six sterile, 125-mL Erlenmeyer flasks containing 1 g (dry weight, 1.18 g wet weight) of untreated soil and 30 mL simulated groundwater were placed in a rotary

shaker at 225 rpm and 25°C for 48 hours. After this time period, the flask contents were transferred into 50-mL tubes for centrifugation at 3,500 rpm for 3 minutes. The soil pellets were resuspended in 30 mL simulated groundwater in sterile 125-mL Erlenmeyer flasks for the mineralization experiment. For each of the six flasks required for testing mineralization of anthracene by bioreactor-treated soil, 14.2 mL of slurry (1 g dry weight) was centrifuged in the same manner as the untreated soil and the soil pellet washed with 30 mL reactor buffer (pH 7.5) with varying ammonium nitrate concentrations (either 5 mM, 2.5 mM, or 1.0 mM). For all incubations, a stock solution of unlabeled anthracene was created by adding 0.0125 g of anthracene to 20 mL dichloromethane (DCM; final concentration 625 µg/mL). One mL of the stock solution was added to each flask prior to adding soil or buffer and the DCM was allowed to evaporate. For mineralization assays, 5 µL of [1,2,3,4,4a,9a-^{14}C]anthracene (approximately 57,000 dpm total) was added to the bottom of each flask prior to adding soil or buffer. In the flasks containing ^{14}C-anthracene, a 13 mm (diameter) x 100 mm glass test tube containing an approximately 2.5-cm square piece of chromatography paper with 60 µL of 2 N KOH was placed into the flask to trap carbon dioxide. This paper was removed daily and replaced with a new piece of chromatography paper spiked with KOH. Removed traps were placed in a scintillation vial along with 5 mL scintillation fluid and the $^{14}CO_2$ was quantified using a Packard Tri-Carb 1900 TR Liquid Scintillation Analyzer.

Samples without ^{14}C-anthracene were used to determine the concentration of anthracene in the flasks at the same time points identified above. One mL was collected at each time point for all samples and placed in a glass, screw-cap test tube with 3 mL

ethyl acetate. The samples were vortexed for 20 seconds and placed in a rotary shaker at 225 rpm and 25°C for 24 hours. After shaking, the samples were left on the bench-top for 15 minutes to settle. The ethyl acetate at the top of the samples was removed using a glass Pasteur pipette, placed in a crimp top 2-mL vial, and stored in the refrigerator. After all samples were collected, they were filtered using a 0.20 µm (pore size) nylon membrane filter. Ten µL of the filtered ethyl acetate extract was placed in an HPLC vial with 990 µL of acetonitrile. These samples were analyzed by high-pressure liquid chromatography (HPLC) using a Waters (Milford, MA) 600E system controller, a Waters 717 Plus autosampler, a Perkin Elmer (Beaconsfield, United Kingdom) LS40 fluorescence detector and a Supelcosil™ LC-PAH column with 3 µm particle size (Supelco, Bellefonte, PA). Filtered samples were analyzed as described previously (Singleton et al. 2008).

C. Stable-Isotope Probing Experiment

Eight sterile, 125-mL Erlenmeyer flasks were equally divided into two groups and spiked with 1 mL of one of two anthracene stock solutions (one with unlabeled anthracene and the other with [U-^{13}C]anthracene) at a concentration of 625 µg/mL in dichloromethane (DCM); the DCM was then allowed to evaporate. One gram dry weight of bioreactor slurry was added to two flasks spiked with ^{13}C-anthracene and two flasks spiked with unlabeled ^{12}C-anthracene. A 30-mL volume of reactor buffer (pH 7.5) containing 5 mM phosphate and 2.5 mM ammonium nitrate was added to each flask. Similarly, 1 gram dry weight of untreated soil that had been previously mixed (method described above) with 30 mL simulated groundwater was added to two flasks spiked with

^{13}C-anthracene and two flasks spiked with unlabeled ^{12}C-anthracene. These eight flasks incubated on a rotary shaker at 225 rpm and 25°C for 5 days.

1. DNA extraction, molecular analysis, and anthracene disappearance

Two mL of soil slurry were removed from flasks containing unlabeled anthracene at 0 h, 24 h, 54 h, 76 h, and 99 h. One mL of the slurry was stored in the freezer (-20 °C) in a 1.5-mL Eppendorf tube for later DNA extraction and molecular analysis and 1 mL was processed following the same procedure for HPLC analysis as described above, except the vortexing time was changed from 20 seconds to 1 minute.

At the end of the five-day SIP experiment, all of the DNA was extracted from the replicate bioreactor-treated soil and untreated soil samples using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH) in four 250-mg aliquots for each of the duplicate ^{13}C-anthracene microcosms and four 162.5-mg aliquots for each of the duplicate unlabeled anthracene microcosms. The published kit protocol was modified by attaching each aliquot in a horizontal fashion to a bench-top vortexer and repeating the protocol on the same aliquot to obtain two DNA extracts for maximum DNA removal (Jones et al. 2011a). The extracted DNA from each extraction for each soil aliquot was stored in 100 µl Tris-EDTA buffer (TE) before further processing. For a given incubation flask, DNA from the first extraction of the four soil aliquots was combined (400 µl total) and DNA from the second extraction of the four soil aliquots was also combined (also 400 µl total). For ultracentrifugation, 300 µl of DNA from each of the two successive extractions per sample was pooled (600 µl total), 2 µl *Escherichia coli* DNA (concentration 75 ng/µl) and 20 µl SYBR Safe (Martineau et al. 2008) were combined with a CsCl solution in TE

to form a final buoyant density (ρ) of 1.72 g/ml in a 6-ml polyallomer Ultracrimp tube (Kendro Laboratory Products, Newtown, CT). These tubes were filled to within 10 mg of each other, crimp sealed, and ultracentrifuged (RC70 ultracentrifuge; Sorvall, Newton, CT) at 175,800 x g and 20°C for 40 hours in a TV-1665 vertical rotor (Sorvall). The ultracentrifuge tubes were examined for DNA presence with a Safe Imager blue light transilluminator (Invitrogen, Carlsbad, CA) prior to fractionation. The ultracentrifuge tubes were fractionated into 24 fractions of 250 µl each, as described by Singleton et al. (2005). DNA was purified and recovered from each fraction through ethanol precipitation as described by Martineau et al. (2008) and resuspended in 50 µl of TE.

2. Identification of fractions containing ^{13}C-enriched DNA

To identify fractions containing ^{13}C-enriched DNA, PCR was performed using two different primer sets. The first was a general bacterial primer set (341F-517R, final concentration 15µM each) and was used to determine the fractions where bacterial DNA was present. The PCR program for those primers consisted of 15 minutes at 94°C, followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 45 seconds at 72°C, and ended with a final dwell at 72°C for 5 minutes. PCR products for bacterial 16S rRNA primers were examined on a 2% agarose gel run for 1 hour at 120V. The second primer set (*E. coli*; final concentration 12.5 µM each) was used to determine the fractions where spiked, unlabeled *E. coli* DNA was present. This PCR program consisted of 3 minutes at 94°C, followed by 25 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 1.5 minutes at 72°C, and ended with a final dwell at 72°C for 3 minutes. PCR products from *E. coli* reactions were examined on a 1% agarose gel run for 1 hour at 120V.

For denaturing-gradient gel electrophoresis (DGGE) analysis of bacterial diversity in each fraction, PCR was performed using primer set 341FGC-517R for four fractions per sample identified as containing heavy DNA (bacterial DNA present but no *E. coli* amplicon detected) and four fractions per sample identified as containing light DNA (where both bacterial DNA and *E. coli* DNA were present). This PCR program consisted of 5 minutes at 94°C, followed by 10 cycles of 1 minute at 94°C, 1 minute at 65°C, and 3 minutes at 72°C, followed by 15 cycles of 1 minute at 94°C, 1 minute at 55°C, and 3 minutes at 72°C and ended with a final dwell of 72°C for 7 minutes. These PCR products were run for 17 hours at 60V and 60°C on a 10% polyacrylamide gel with a linear gradient of denaturants (formamide and urea) from 35% to 65% with a non-denaturing stacking gel on a DCode system (Bio-Rad Laboritories, Hercules, CA).

DNA was quantified in each collected fraction from ultracentrifuge tubes using a NanoDrop ND-3300 fluorospectrometer with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR).

3. Cloning of 16S rRNA genes from heavy DNA

Once fractions containing ^{13}C-enriched DNA were identified for a given sample, they were combined (1 µl DNA from a pool of 2 µl of DNA per heavy fraction) for 16S rRNA gene cloning and sequencing of the cloned genes. The PCR for cloning was performed using universal bacterial set 8F and 1492R (PCR program: 15 minutes at 95°C, followed by 25 cycles of 1 minute at 94°C, 1 minute at 50°C, and 3 minutes at 72°C, ending with a final dwell at 72°C for 15 minutes) in a final volume of 50 µl. Separate PCR reactions were performed for samples from the duplicate incubations. PCR products were examined on a 1% agarose gel with DNA standard λHindIII to verify the

presence and quality of DNA. Three μL of the product was cloned using a TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and plated on an LB agar medium containing kanamycin. After 24 h, 35 colonies per sample (70 colonies total) were picked and inoculated into 5 mL LB broth (Invitrogen, Carlsbad, CA) with kanamycin. Of these clones, 24 from each sample (48 total) were sequenced by Eton Biosciences (Research Triangle Park, Durham, NC) using primers M13F and M13R. Sequences were manually trimmed and assembled using Sequencher 5.0. The sequences were aligned with close relatives determined by BLASTn and a neighbor-joining tree was constructed using ClustalX2. The tree was bootstrapped 1,000 times and gaps were not considered.

4. Quantification of identified bacteria

Cloned sequences from ^{13}C-enriched DNA fractions were grouped into operational taxonomic units (OTUs) at a criterion of greater than 97% similarity, and a representative sequence was identified from each OTU. Representative sequences were identified by aligning all clones in a defined OTU, clustering them and running them through the Ribosomal Database Project's representative sequence function (Cole et al. 2009). Five OTUs containing at least 2 clones were identified as possible anthracene degraders. One OTU was previously identified as "pyrene group 2" (PG2), for which PCR primers have already been designed (Singleton et al. 2006). Primers suitable for quantitative real-time PCR (qPCR) for the other OTUs were developed and tested as previously described (Singleton et al. 2006).

To determine the optimal annealing temperature of developed qPCR primer pairs, representative clones were linearized using PstI, purified with a Nucleotide Purification

Kit (Qiagen), and a conventional PCR assay using a temperature-gradient was run. A standard curve for the quantification of each representative sequence was created by performing a serial dilution of the linearized plasmid template in water and running PCR reactions on a SmartCycler (Cepheid) machine. The concentration of DNA in the serial dilutions was measured using NanoDrop as described above. The r^2 value for each qPCR standard curve was ≥ 0.995.

Primer specificity was tested by assessing cross-amplification of a given primer set to the other putative anthracene-degrading OTU representative sequences by qPCR. In addition, gene abundance of DNA samples extracted from slurry samples taken over the course of the SIP incubation was measured for all OTUs.

IV. Results

A. Preliminary Experiments

1. Screening colonies for "anthracene group 1"

In an attempt to isolate previously identified anthracene-degrading bacteria known as "anthracene group 1" (Jones et al. 2011a), untreated soil samples enriched with anthracene in simulated groundwater was spread on a modified 216L and R2A agar plates. A modified 216L agar was used (simulated groundwater replaced seawater) because it was used to cultivate a marine *Altererythobacter* strain that was closest isolated relative to AG1. Over 50 individual colonies were screened but none yielded positive amplicon when tested by PCR using AG1-specific primers.

2. "Anthracene group 1" and pH effects in bioreactor-treated soil

The effect of biostimulation of untreated, PAH-contaminated soil and bioreactor-treated soil from a former MGP site in Salisbury, NC on the abundance of 16S rRNA genes associated with the previously identified bacterial "anthracene group 1" (AG1) was tested using two different buffers amended with anthracene. Simulated groundwater amended with low concentrations of nitrogen (N) and phosphorous (P) was compared to reactor buffer containing much higher concentrations of N and P. After 114 h of incubation, the total bacterial 16S rRNA gene quantity varied by less than an order of magnitude under all conditions (Figure 5). In contrast, the abundance of AG1 16S rRNA genes changed significantly depending on the soil source and the buffer (Figure 6). The number of AG1 16S rRNA genes in the untreated soil in both the simulated groundwater and reactor buffer incubations increased at least an order of magnitude for both buffers after 114 h. In contrast, AG1 16S rRNA genes in the bioreactor-treated soil declined by more than an order of magnitude in both simulated groundwater and reactor buffer, suggesting that AG1 was likely not a dominant anthracene degrader in the bioreactor.

The pH of the various incubations was measured over time (data shown in Figure 5 andFigure 6). The pH of incubations with untreated soil in both simulated groundwater and reactor buffer declined from 8.06 at 0 h to 7.03 and 7.09, respectively, at 114 h. The bioreactor-treated soil in simulated groundwater had an initial pH of 7.53, which decreased to 7.02 at 114 h, whereas in the reactor buffer, the pH decreased to 5.37 at 114 h. Such a drop in pH has been observed previously for the treatment of the Salisbury MGP soil in the bioreactor as well as in batch incubations, such as those utilized in this experiment (Singleton et al. 2011).

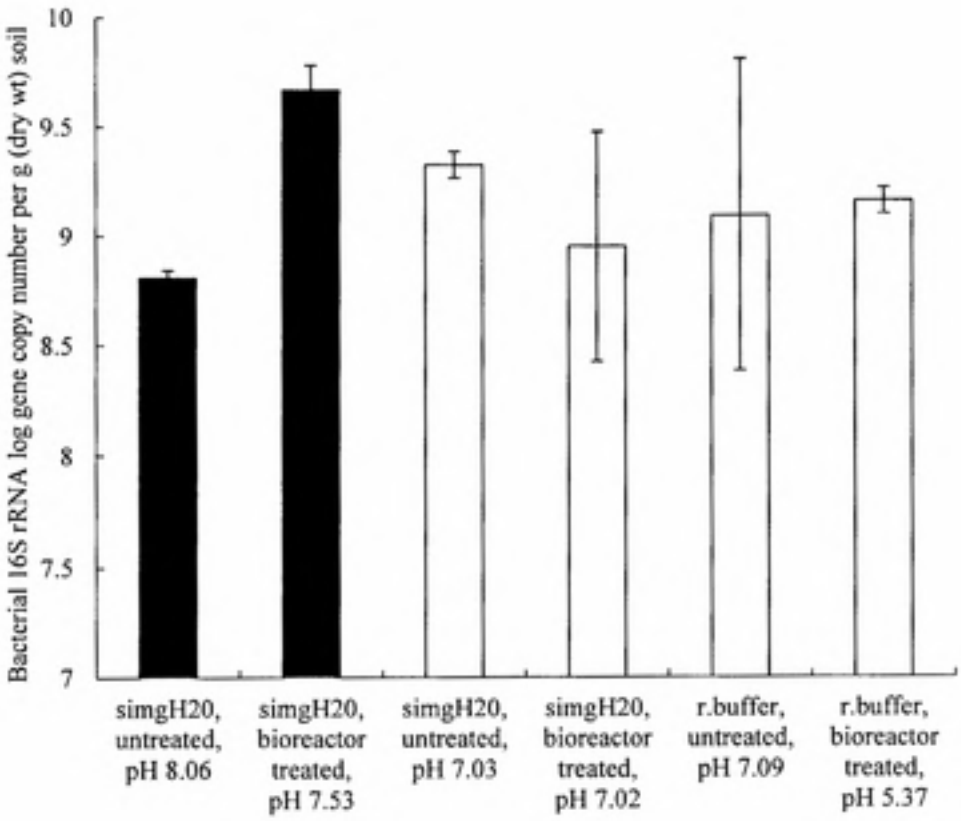


Figure 5: Abundance of bacterial 16S rRNA genes with different buffers and soil samples (untreated soil or slurry from the bioreactor) at 0 h (filled bars) and 114 h (open bars), with pH measurements. SimgH20, simulated groundwater amended with N and P; r.buffer, reactor buffer. Data represent mean and standard deviations of triplicate incubations for each condition.

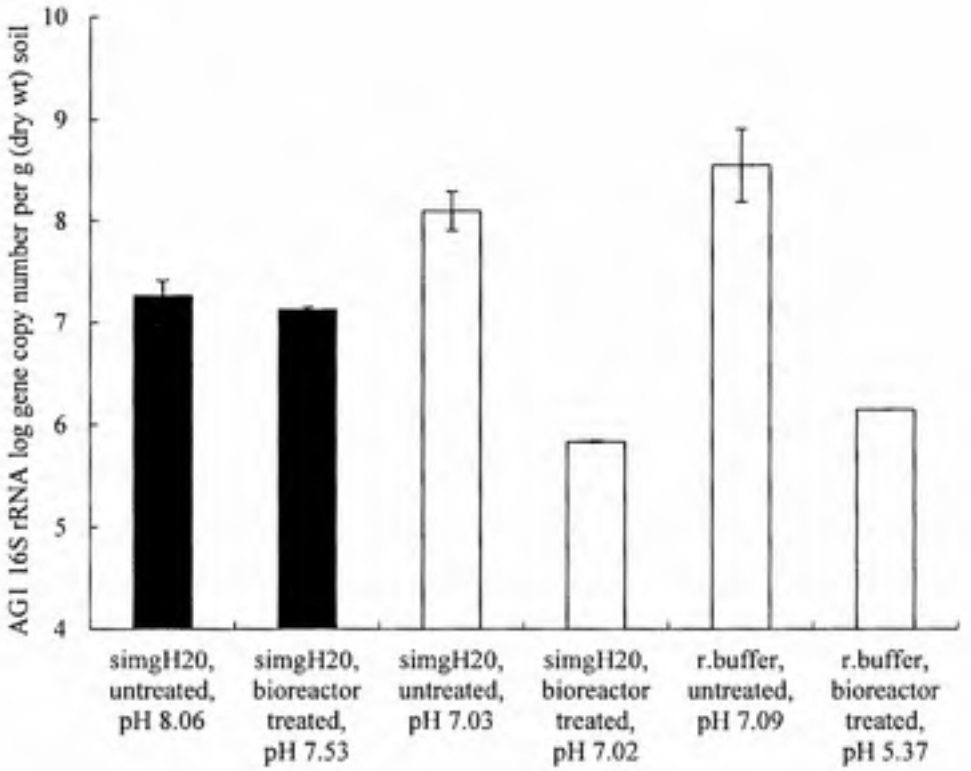


Figure 6: Abundance of AG1 16S rRNA genes with different buffers and soil samples (untreated soil or slurry from the bioreactor) at 0 h (filled bars) and 114 h (open bars), with pH measurements. SimgH20, simulated groundwater amended with N and P; r.buffer, reactor buffer. Data represent mean and standard deviations of triplicate incubations for each condition.

3. Effect of incubation medium on pH of the bioreactor-treated soil

To test if the incubation medium had an effect on the pH of the bioreactor-treated soil, samples were washed five times in either simulated groundwater or reactor buffer, incubated, and then bacterial and AG1 16S rRNA gene abundance was quantified and the pH measured. After washing each sample five times in a given buffer, the abundance of bacterial 16S rRNA genes and AG1 16S rRNA genes dropped similarly regardless of the buffer (Figure 7 andFigure 8). The change in pH, however, was dependent on the buffer and was even more pronounced when the reactor slurry was washed with reactor buffer,

dropping to a pH of 4.78 at 121 h, than when washed only once (pH of 5.37 at 114 h) as shown in Figure 5.

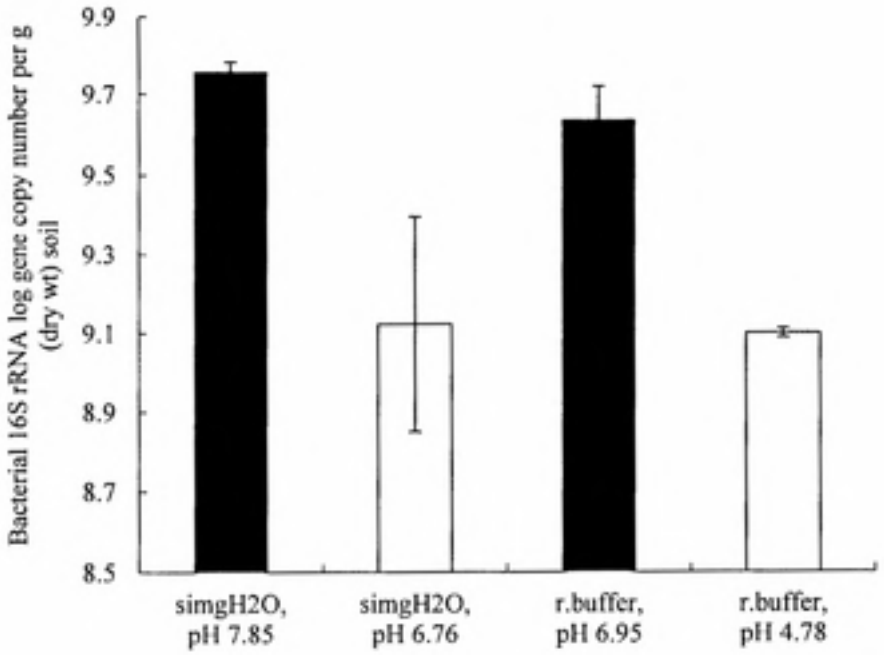


Figure 7: Abundance of bacterial 16S rRNA genes from bioreactor-treated soil with different buffers at 0 h (filled bars) and 121 h (open bars) after washing 5 times, with pH measurements. SimgH20, simulated groundwater amended with N and P; r.buffer, reactor buffer. Data represent mean and standard deviations of triplicate incubations for each condition.

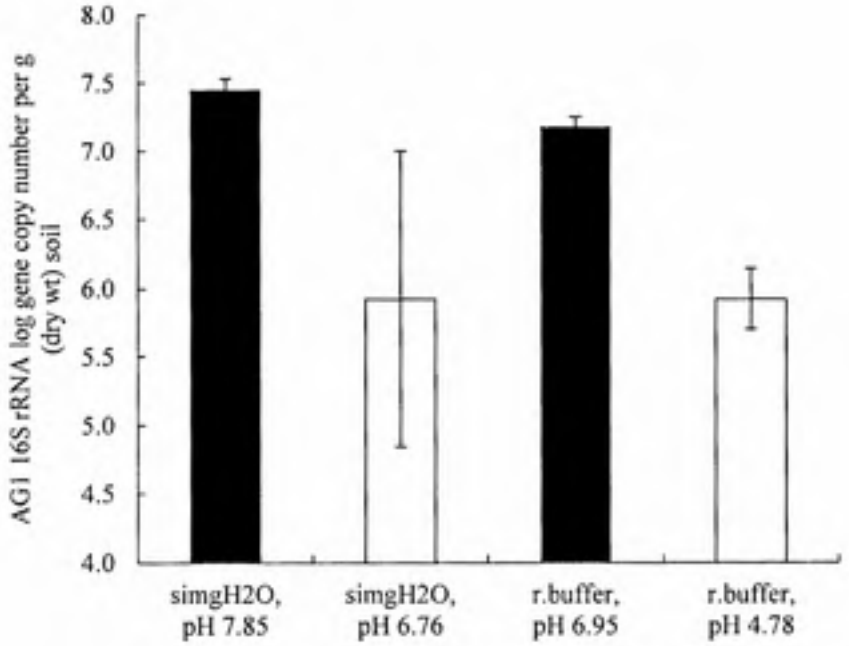


Figure 8: Abundance of AG1 16S rRNA genes from bioreactor-treated soil with different buffers at 0 h (filled bars) and 121 h (open bars) after washing 5 times, with pH measurements. SimgH20, simulated groundwater amended with N and P; r.buffer, reactor buffer. Data represent mean and standard deviations of triplicate incubations for each condition.

4. Effects of ammonium nitrate concentration on slurry pH, mineralization, and anthracene disappearance

The results reported above indicated that the reactor buffer used was likely causing the pH drop observed during incubations with bioreactor-treated soil. On the assumption that nitrification was leading to decreased pH, the effects of varying ammonium nitrate concentrations (1 mM, 2.5 mM, and 5.0 mM) on pH, ^{14}C-anthracene mineralization rates, and anthracene disappearance was measured. Results for pH are shown in Figure 9.

When washed one time with phosphate buffer containing 5 mM ammonium nitrate, the bioreactor-treated soil had a pH of 7.03 ± 0.02 at 0 h, but after 114 h of incubation, the pH decreased to 5.37 ± 0.1. In contrast, incubations for 117 h with either 1 mM or 2.5 mM ammonium nitrate did not lead to a substantial change in pH (Figure 9).

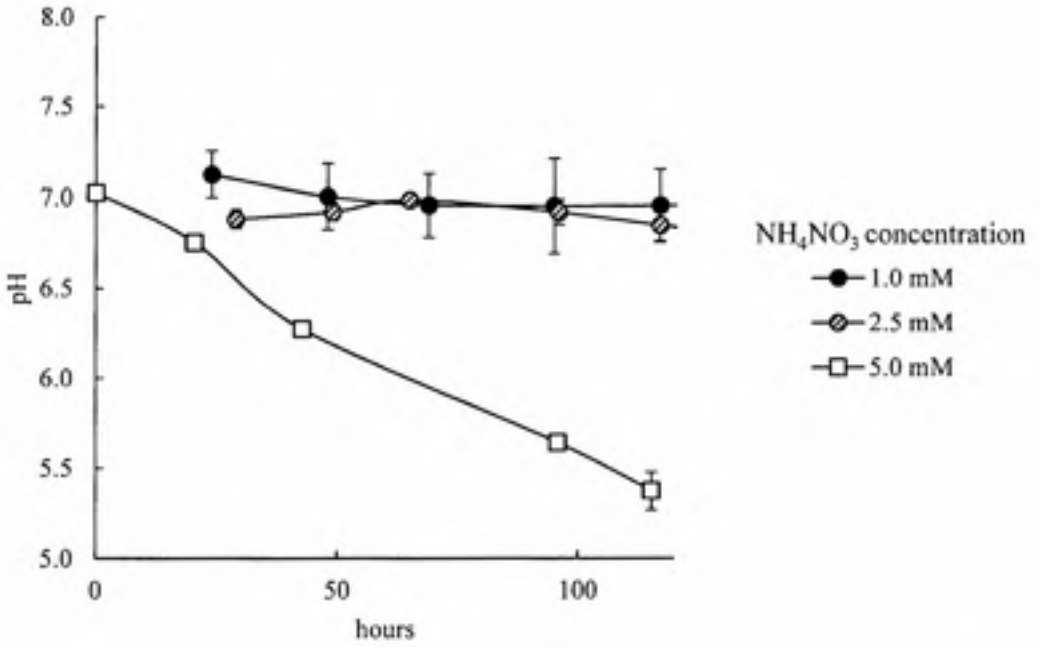


Figure 9: pH measurements over time for incubations of reactor slurry in 5 mM phosphate buffer containing different concentrations of ammonium nitrate.

Incubations with the reactor buffer containing the original 5.0 mM ammonium nitrate resulted in 30.5% of the added ^{14}C-anthracene being mineralized after 114 h (Figure 10). This compares with 22.9% of the added ^{14}C-anthracene being mineralized in 117 h when 2.5 mM ammonium nitrate was used and 2.2% of the added ^{14}C-anthracene being mineralized in 117 h when 1.0 mM ammonium nitrate was used.

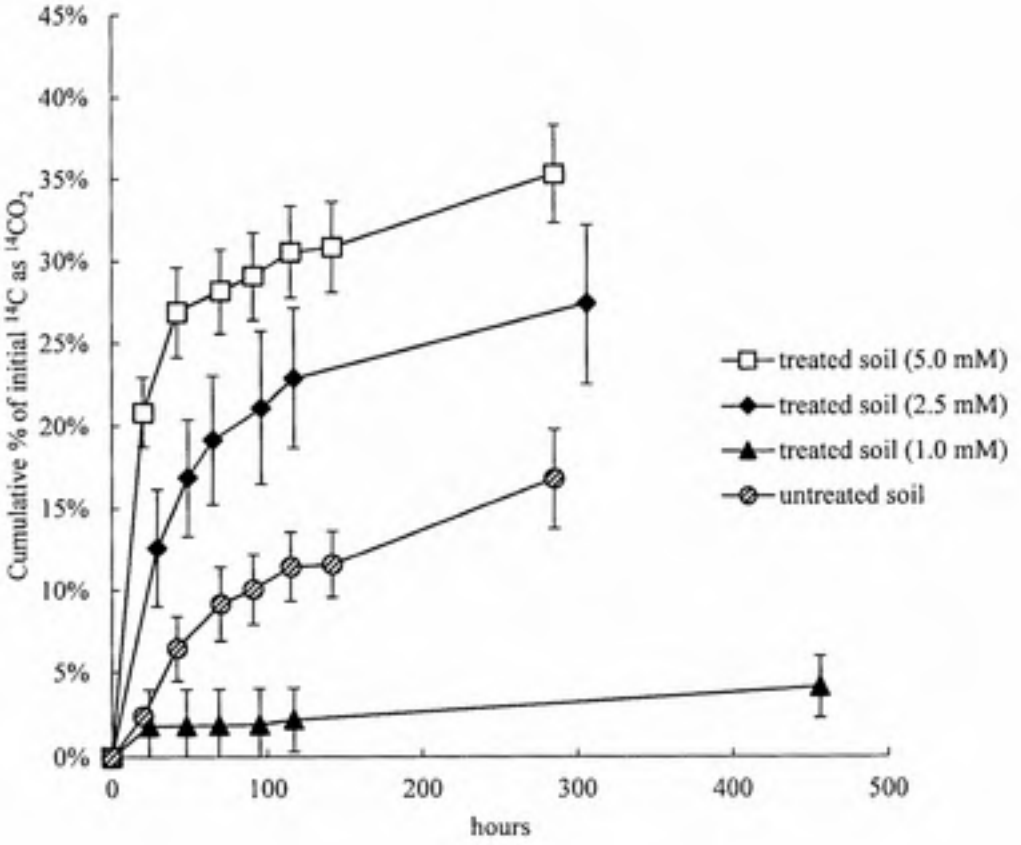


Figure 10: Cumulative $^{14}CO_2$ recovered from incubations of bioreactor-treated soil with [14C]anthracene in 5 mM phosphate buffer containing different concentrations of ammonium nitrate; NH_4NO_3 concentration is shown in parentheses in the legend. Results from incubations of untreated soil with simulated groundwater amended with N and P are shown for comparison.

The concentration of unlabeled anthracene in flasks incubated in tandem with the mineralization experiment was determined by HPLC analysis. The nominal initial concentration of anthracene in the flasks was 625 µg, or 21 µg/mL. The amount of ammonium nitrate added to the phosphate buffer did not appear to influence anthracene disappearance (Figure 11).

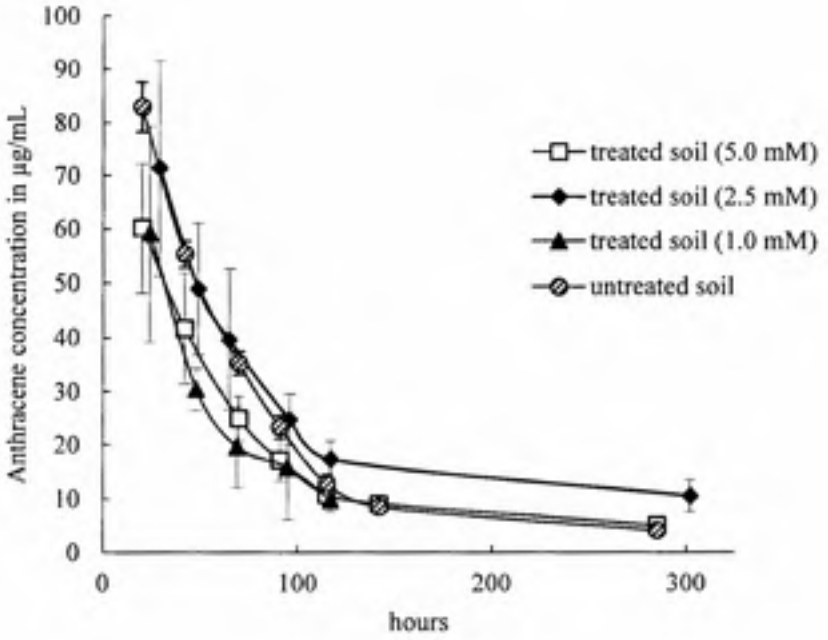


Figure 11: Anthracene disappearance over time with bioreactor-treated soil incubated in 5 mM phosphate buffer at different ammonium nitrate concentrations. Results from incubations of untreated soil in simulated groundwater amended with N and P are shown for comparison.

Using information from pH measurements, ^{14}C-anthracene mineralization rates, and anthracene disappearance it was concluded that the SIP experiment with ^{13}C-anthracene would be performed with phosphate buffer containing 2.5 mM ammonium nitrate. Based on the mineralization and anthracene disappearance experiments, an incubation period of five days (124 hours) was selected for SIP incubations.

B. Stable-Isotope Probing Experiment

1. Anthracene disappearance

Duplicate flasks containing bioreactor-treated soil or untreated soil were incubated in phosphate buffer containing 2.5 mM ammonium nitrate or simulated

groundwater, respectively, and 625 µg of either unlabeled anthracene or ^{13}C-anthracene for SIP. The concentration of unlabeled anthracene was measured over the five days of the incubation. Unlike prior tests, which indicated 65% anthracene disappearance in 96 h using bioreactor-treated soil with 2.5 mM ammonium nitrate phosphate buffer (Figure 11), only 15% of the anthracene disappeared after 99 h in the SIP study (Figure 12). For untreated soil with simulated groundwater, anthracene removal was 72% after 91 h in the preliminary experiment (Figure 11) but only 52% in the SIP experiment after 99 h (Figure 12). Despite the disappearance in anthracene concentrations between preliminary and SIP experiments, heavy DNA was recovered in the SIP experiment.

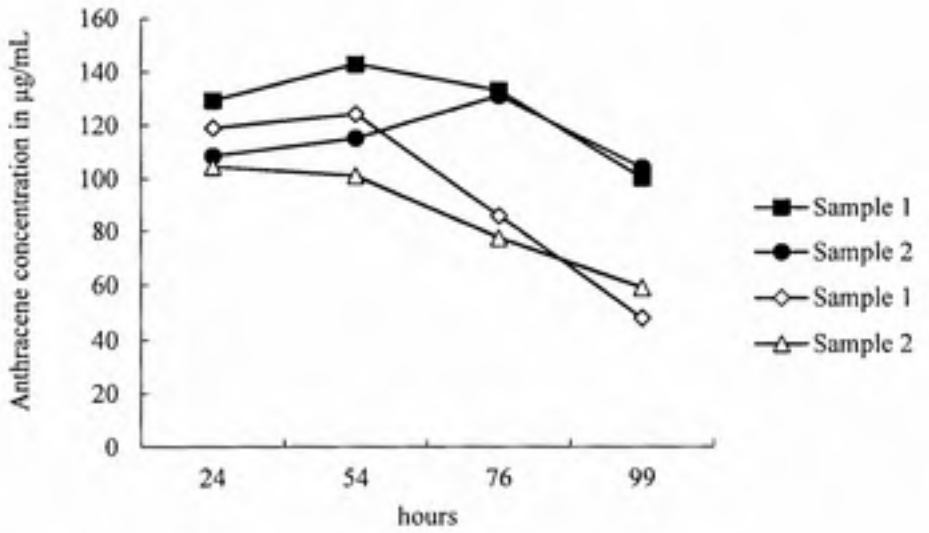


Figure 12: Anthracene disappearance over time in the SIP experiment for duplicate incubations of bioreactor-treated soil and phosphate buffer containing 2.5 mM ammonium nitrate (filled symbols) or untreated soil incubated in simulated groundwater amended with N and P (open symbols).

2. Identification of fractions containing ^{13}C-enriched DNA

After ultracentrifugation of the DNA from the untreated soil in incubations with ^{13}C-anthracene, there was no evidence of ^{13}C-enriched DNA either through visual examination of the ultracentrifugation tubes or during PCR analyses of collected fractions. The untreated soil was therefore excluded from additional analysis in this study.

After DNA extracted from the bioreactor-treated soil (slurry) incubated in the presence of ^{13}C-labeled anthracene was separated by ultracentrifugation and fractioned, a number of steps were taken to identify fractions containing ^{13}C-enriched DNA. These included DNA quantification, screening for spiked *E. coli* DNA (which was unlabeled), and DGGE analysis of the purified fractions. Incubations containing [U^{13}C]-labeled anthracene produced a positive product for 16S rRNA genes via conventional PCR in fractions where no products appeared in equivalent fractions obtained from incubations with unlabeled anthracene; these were, therefore, identified as the heavy fractions. The addition of *E.coli* DNA prior to ultracentrifugation allowed for a normalization of the light and heavy fraction locations for samples from both the labeled and unlabeled anthracene incubations. Based on the lowest (i.e., highest density) fraction containing PCR-amplifiable *E. coli* 16S rRNA genes, fractions 7-10 of the incubations derived from [U^{13}C]-labeled anthracene were identified as 'heavy' while fractions lower than 11 were considered 'heavy' for incubations with unlabeled anthracene (Figure 13 and Figure 14). DGGE was used to confirm heavy and light fraction separation.

DNA quantification was performed on both the labeled and unlabeled anthracene incubations. The amount of DNA extracted was variable between duplicate samples,

particularly for the incubations with unlabeled anthracene (Figure 16). The presence of DNA in the 'heavy' fractions from each sample is shown more clearly in (Figure 17). Sample 2 from the ^{12}C extraction, however, had more DNA in the 'heavy' fractions, where DNA was not expected, than did sample 1. This could have been caused by the greater amount of DNA in sample 2 than in sample 1 from the incubations with unlabeled anthracene, and not because of an actual increase in DNA density in sample 2.

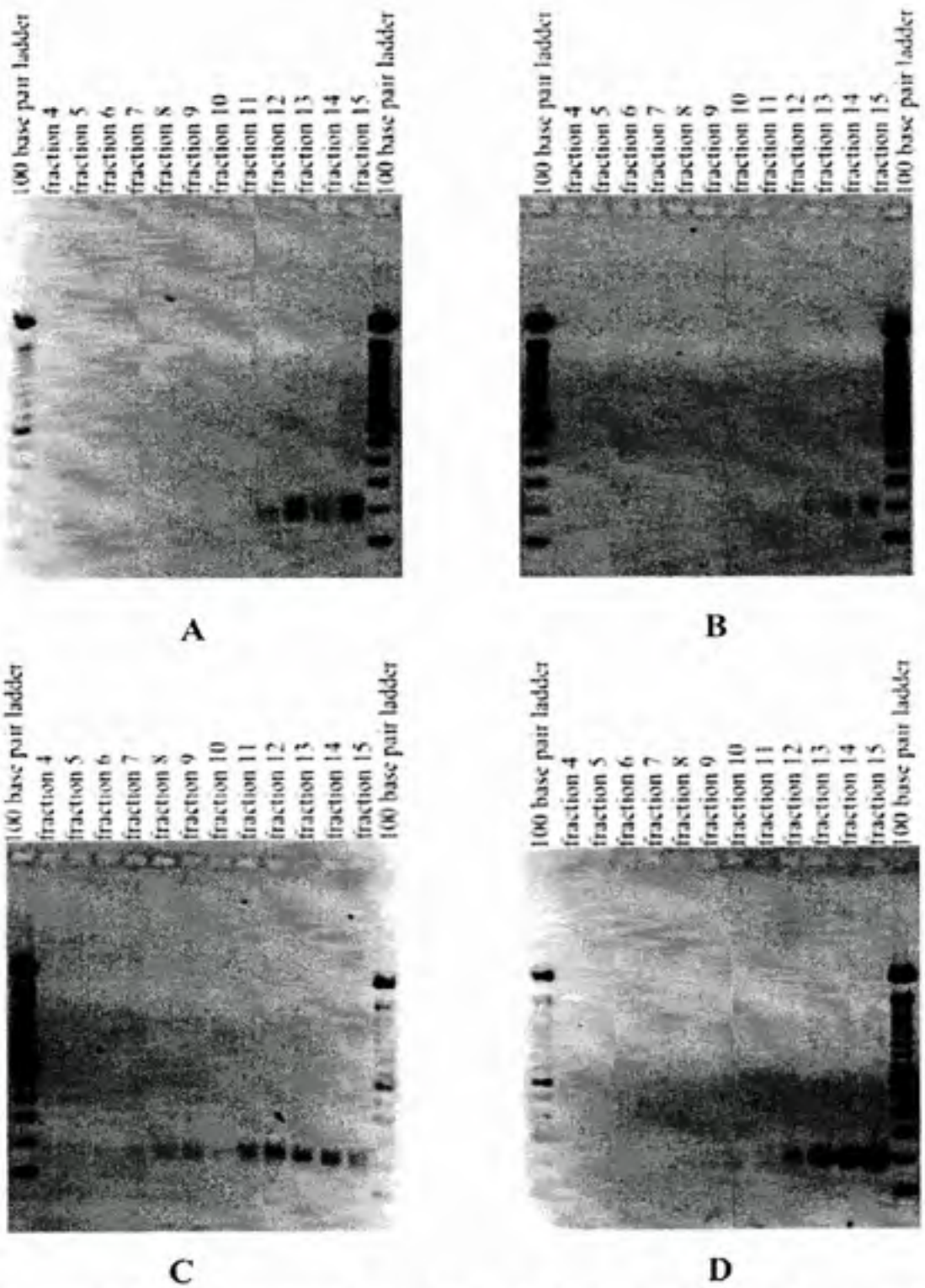


Figure 13: General bacterial 16S rRNA gene PCR analyses of collected fractions. **A**: ^{12}C slurry sample 1, fractions 4-15; **B**: ^{12}C slurry sample 2, fractions 4-15; **C**: ^{13}C slurry sample 1, fractions 4-15; **D**: ^{13}C slurry sample 2, fractions 4-15. In all cases, fractions are numbered from the bottom of the ultracentrifuge tubes (fraction 1) to the top of the tubes (fraction 15).

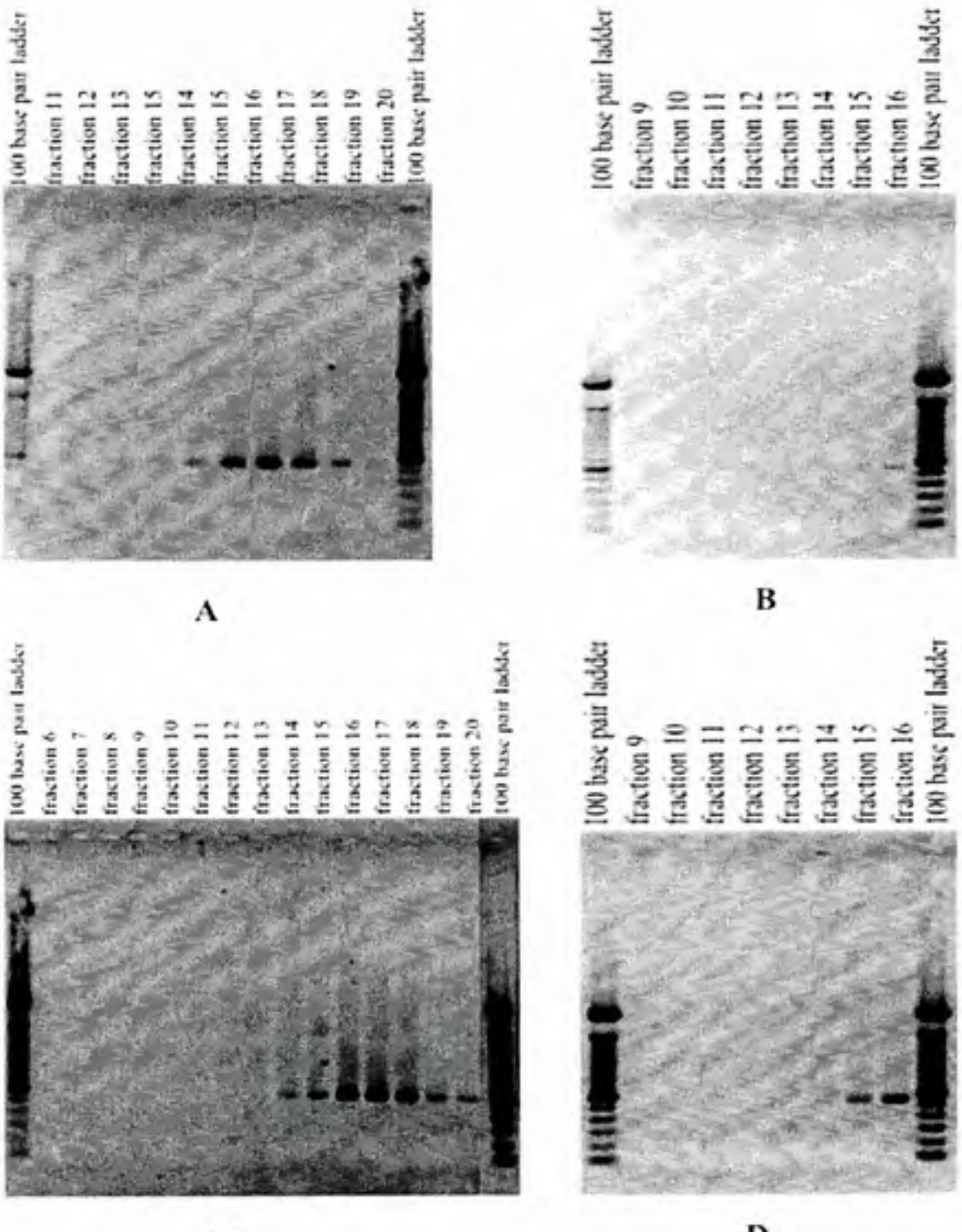


Figure 14: PCR products from reactions with *E.coli*-specific primers. **A**: ^{12}C slurry sample 1, fractions 11-20; **B:** ^{12}C slurry sample 2, fractions 9-16; **C:** ^{13}C slurry sample 1, fractions 9-16; **D:** ^{13}C slurry sample 2, fractions 9-16. In all cases, fractions are numbered from the bottom of the ultracentrifuge tubes (fraction 1) to the top of the tubes (fraction 15).

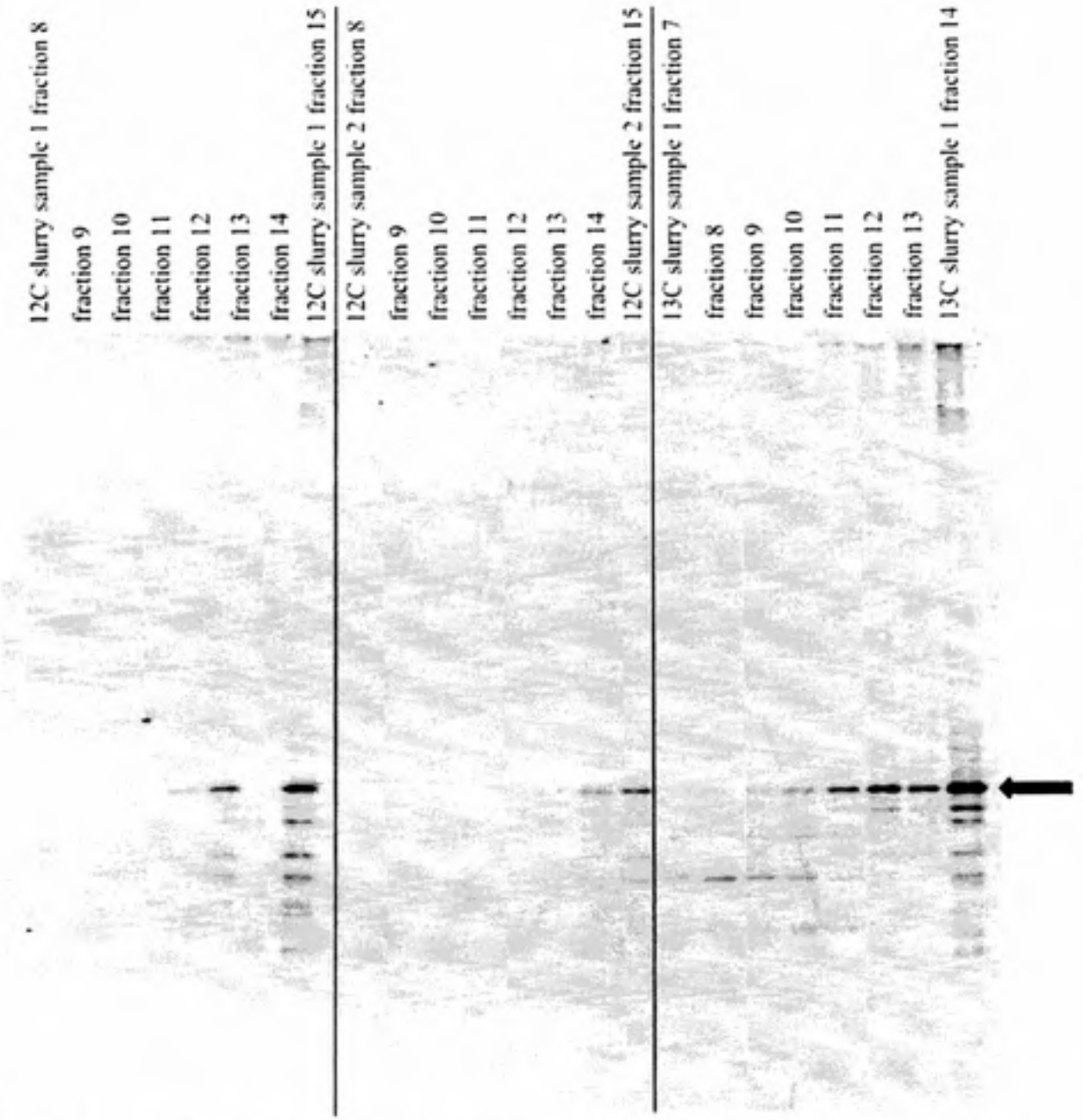


Figure 15: DGGE of 'light' and 'heavy' fractions of ^{12}C slurry, samples 1 and 2, and ^{13}C slurry sample 1 showing *E.coli* 16S rRNA genes band in 'light' fractions.

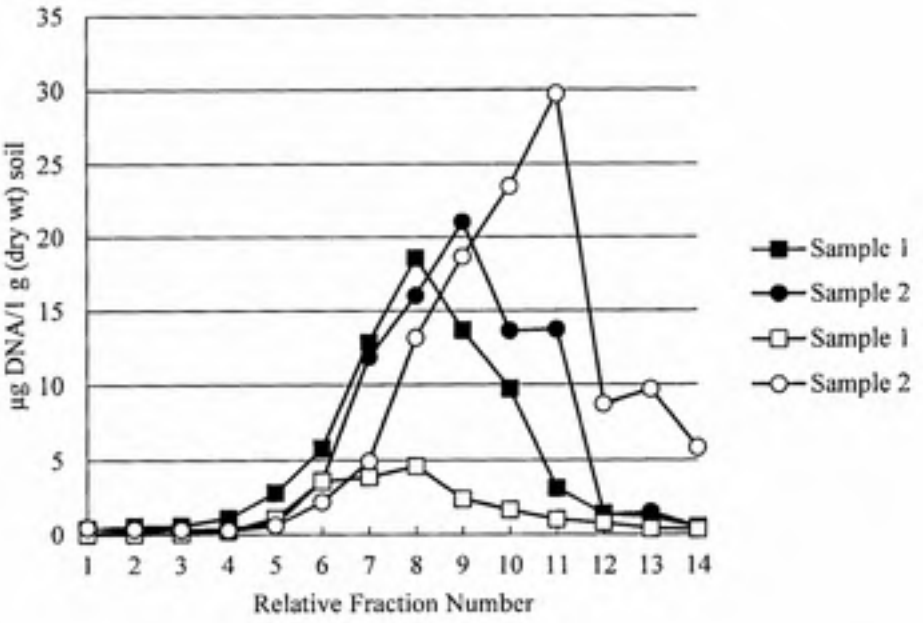


Figure 16: Quantification of DNA from two consecutive extractions from incubations with ^{13}C-anthracene (filled symbols), and unlabeled anthracene (open symbols). Relative fractions 2 through 5 were considered to contain 'heavy' DNA.

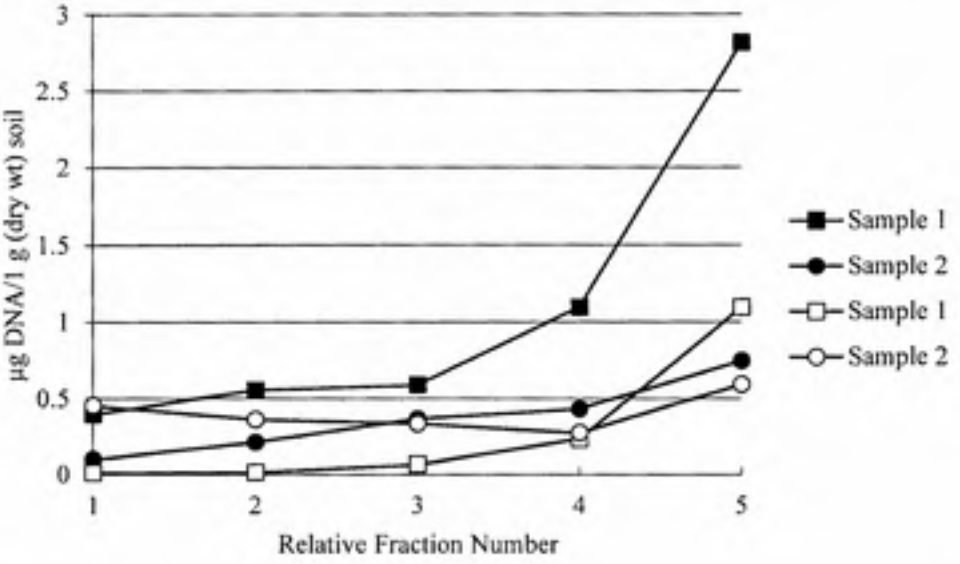


Figure 17: Quantification of DNA from two consecutive extractions from incubations with ^{13}C-anthracene (filled symbols), and unlabeled anthracene (open symbols) for relative fractions 1-5 only. Relative fractions 2 through 5 were considered to contain 'heavy' DNA.

3. Phylogenetic analysis of cloned 16S rRNA genes from ^{13}C-enriched fractions

^{13}C-Enriched DNA from heavy fractions was used to create a 16S rRNA gene clone library totaling 48 clones. The sequences were manually trimmed and assembled using Sequencher 5.0. Only 43 clones possessed sequences of sufficient quality or length to be retained for further analysis. The close relatives of those sequences were determined by BLASTn and a neighbor-joining tree was constructed using ClustalX2 (Figure 18). The combining of sequences into OTUs based on 97% similarity produced five OTUs containing two or more clones and five singleton sequences. Singleton sequences were not included in further analysis. The five OTUs containing two or more clones were designated as OTU-41 (6 clones), OTU-11 (20 clones), OTU-12 (7 clones), OTU-19 (3 clones), and OTU-6 (2 clones) based on the name of their representative sequence. OTU-41 was very closely related to sequences from the previously described PG2 clade (soil clone PYR10d11 in Figure 18), and therefore is referred to as PG2 in subsequent discussion.

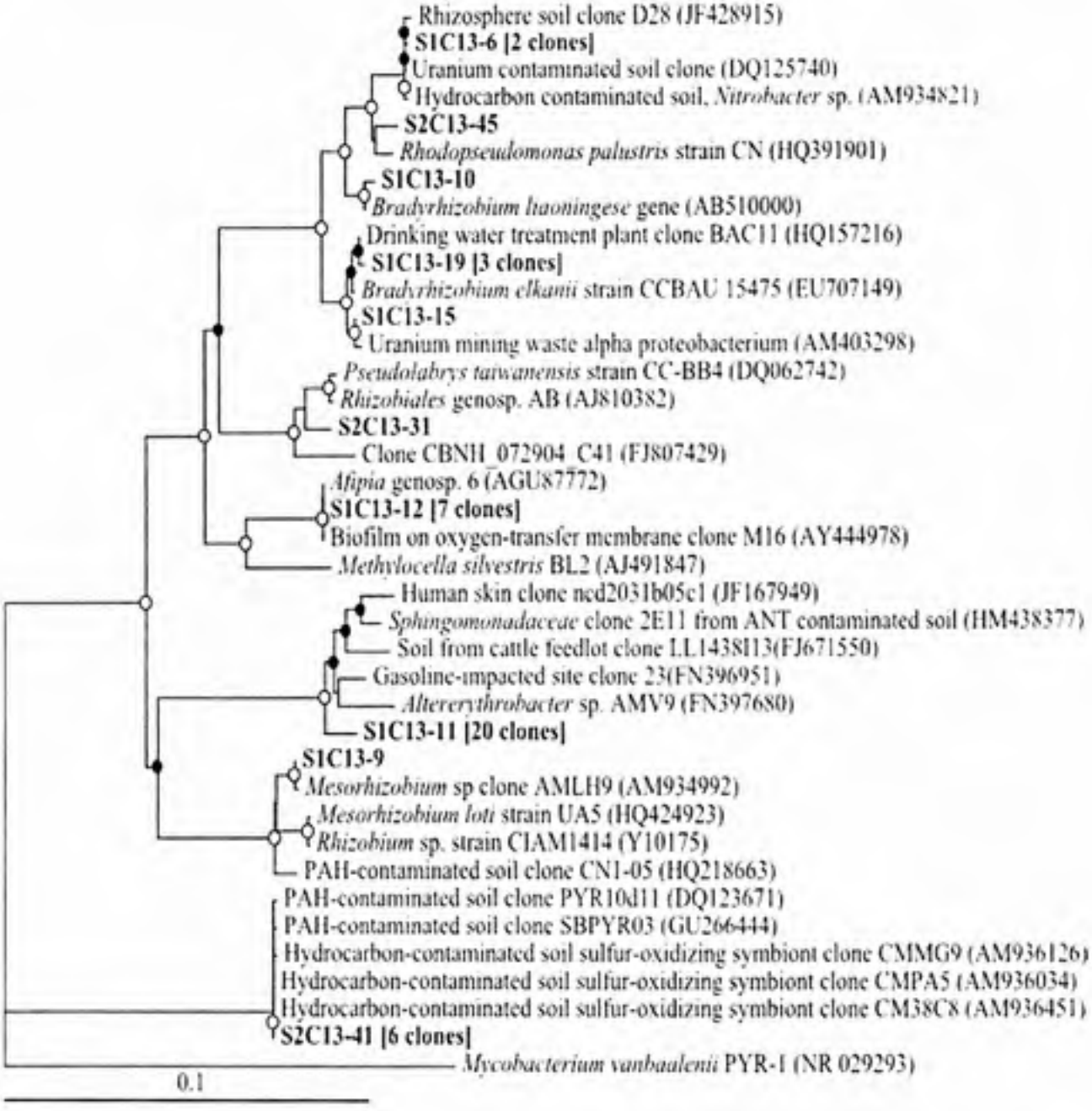


Figure 18: Phylogenetic tree of partial 16S rRNA gene sequences of SIP-identified anthracene-degrading bacteria. Closed and open circles at nodes indicate ≥ 50% and ≥ 95% bootstrap support, respectively.

4. Quantification of identified bacteria

To measure the abundance of the identified OTUs in heavy DNA, primers were developed for qPCR targeting each of the five OTUs separately (Table 1). These primers were used to measure abundance at the end of the SIP incubation as well as growth over the course of the SIP incubation.

Target Group	Primer Name	Sequence 5'→3'	Amplicon Size (bp)	Annealing Temp (°C)	Reference	Mismatch	RDP Hits	Most Hit
Pyrene Group 2 6 clones	GP2.4F GP2.4R	CCA AGC CGA CGA CGG GTA G TTC CCC ACT GCT GCC TC	94	59	Jones, 2011		900	
Group 11 20 clones	OTU11F OTU11R	CGG ATT ACA GAG ATG TTT TC TAG AGT TCC CAA CTG AAT GA	149	56	this study	0 1 2	0 0 568	- - 406, *Sphingominadaceae*
Group 12 7 clones	OTU12F OTU12R	AAA TCC CAG AGC TCA ACT CT CGC AGT TCC ACT TAC CTC TT	75	58	this study	0 1	5 1258	5, *Rhizobiales* 1218, *Rhizobiaceae*
Group 19 3 clones	OTU19F OTU19R	ACA TCC CGG TCG CGG ACT ACC YGT CTC CGG TCC AG	63	60	this study	0	1670	1617, *Bradyrhizobium*
Group 6 2 clones	OTU6F OTU6R	AAC AAC TGA GGG AAA CTT CA TTG GTG AGC CAT TAC CTC	113	57.5	this study	0	1118	1026, *Bradyrhizobiaceae*

Table 1: qPCR primers designed and used in this study

a) PG2

Primers previously developed (Jones et al. 2011b) for quantifying 16S rRNA genes associated with organisms designated "pyrene group 2" (PG2) were used to examine the distribution of genes from these organisms among fractions containing detectable DNA, including those identified as 'heavy' according to previous determinations. The majority of the genes associated with PG2 were not found in the heavy fractions of the sample from incubations with ^{13}C-anthracene (Figure 19) and therefore was not considered an anthracene degrader under the conditions tested. The replicate samples were not tested for this group.

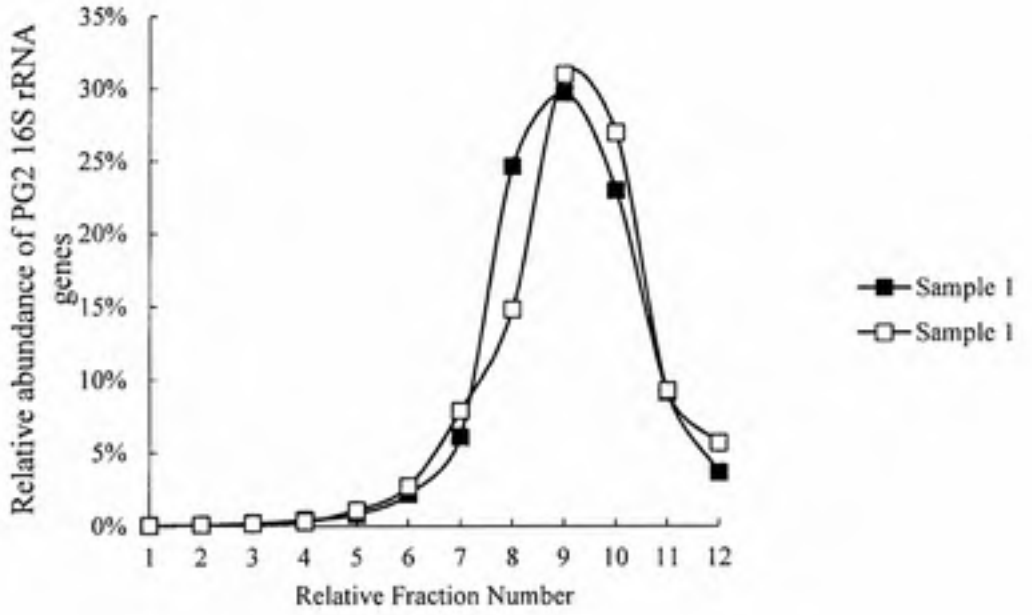


Figure 19: Distribution of PG2 16S rRNA genes in previously identified 'heavy' and 'light' relative fractions from incubations with ^{13}C-anthracene (filled symbols), and unlabeled anthracene (open symbols). Points represent the percentage of genes from that OTU present in each fraction as a percentage of the total genes determined for all displayed fractions. Relative fractions 2 through 5 were considered to contain 'heavy' DNA.

b) OTU-11

Members of OTU-11 were most closely related to *Altererythrobacter* and clones associated with this OTU represented 20 out of 43 clones in the libraries constructed from DNA recovered from heavy fractions. The classifier function on the Ribosomal Database Project (RDP-II) identified OTU-11, with an 80% confidence threshold, to be 100% within the *Sphingomonadales* family and 81% within the genus *Altererythrobacter*.

Based on conventional PCR with a linearized plasmid harboring the representative 16S rRNA gene of the OTU, it was determined that the optimum annealing temperature for the PCR primers developed to quantify OTU-11 was 56°C (Figure 20). A qPCR screen of a range of fractions concluded that a significant percentage of 16S rRNA genes associated with OTU-11 (57%) appeared in the heavy fractions of samples from incubations with ^{13}C-anthracene (Figure 21), whereas OTU-11 sequences were not detected in the relative heavy fractions of the samples from incubations with unlabeled anthracene.

The distribution of genes from OTU-11 in fractions derived from unlabeled anthracene incubations was restricted primarily to fractions associated with unenriched DNA. These data suggest that members of OTU-11 likely incorporated ^{13}C from anthracene or a metabolite of anthracene degradation.

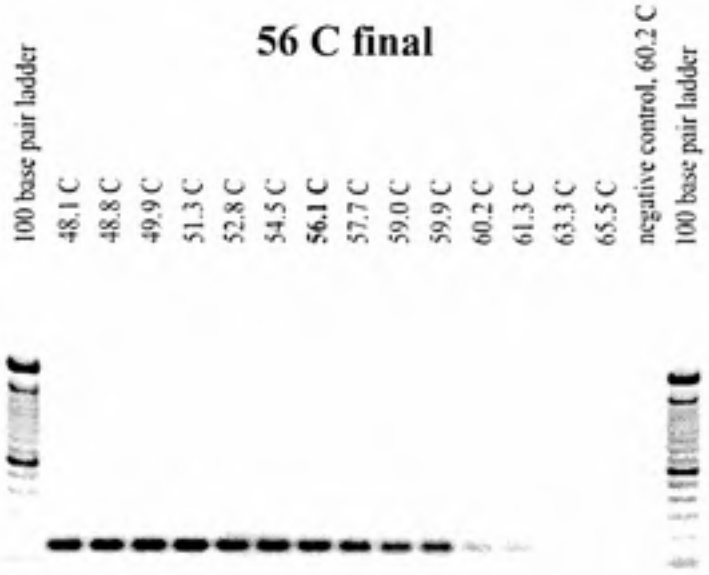


Figure 20: PCR products from OTU-11 digested plasmid and designed qPCR primers at various temperatures.

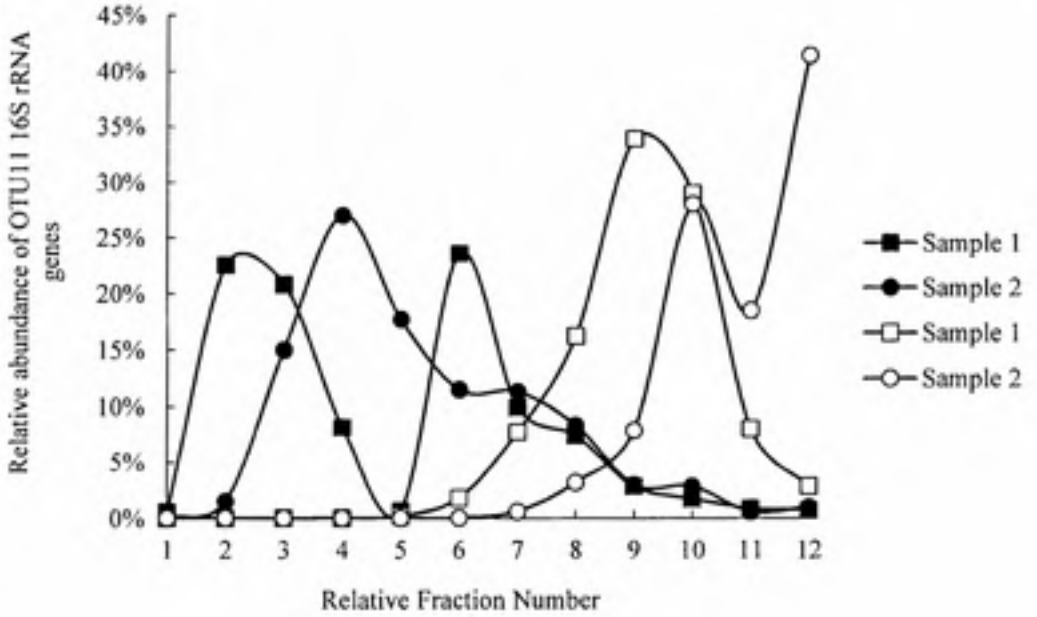


Figure 21: Distribution of OTU-11 16S rRNA genes in previously identified 'heavy' and 'light' relative fractions from incubations with ^{13}C-anthracene (filled symbols), and unlabeled anthracene (open symbols). Points represent the percentage of genes from that OTU present in each fraction as a percentage of the total genes determined for all displayed fractions. Relative fractions 2 through 5 were considered to contain 'heavy' DNA.

c) OTU-12

Members of OTU-12 were identified by RDP-II to be in the order *Rhizobiales* with 100% confidence. Seven of the 43 clones in the libraries constructed from DNA recovered from heavy fractions were associated with this OTU.

Based on conventional PCR with a linearized plasmid harboring the representative 16S rRNA gene of the OTU, it was determined that the optimum annealing temperature for the PCR primers developed to quantify OTU-12 was 58°C (Figure 22). A qPCR screen of a range of fractions concluded that a large percentage of 16S rRNA genes associated with OTU-12 (36%) appeared in the heavy fractions of the samples from incubations with ^{13}C-anthracene (Figure 23), whereas no OTU-12 sequences appeared in the relative heavy fractions of the samples from incubations with unlabeled anthracene.

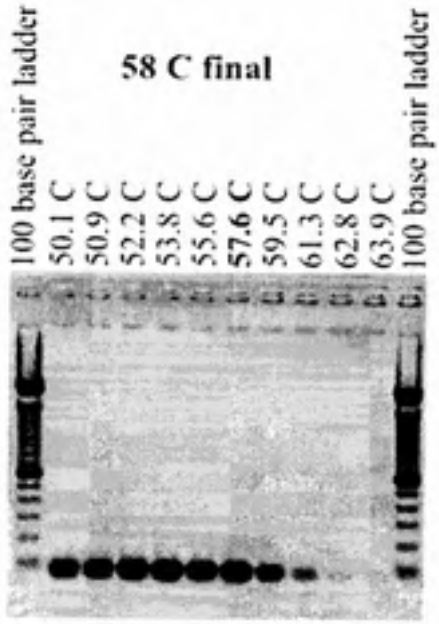


Figure 22: PCR products from OTU-12 digested plasmid and designed qPCR primers at various temperatures.

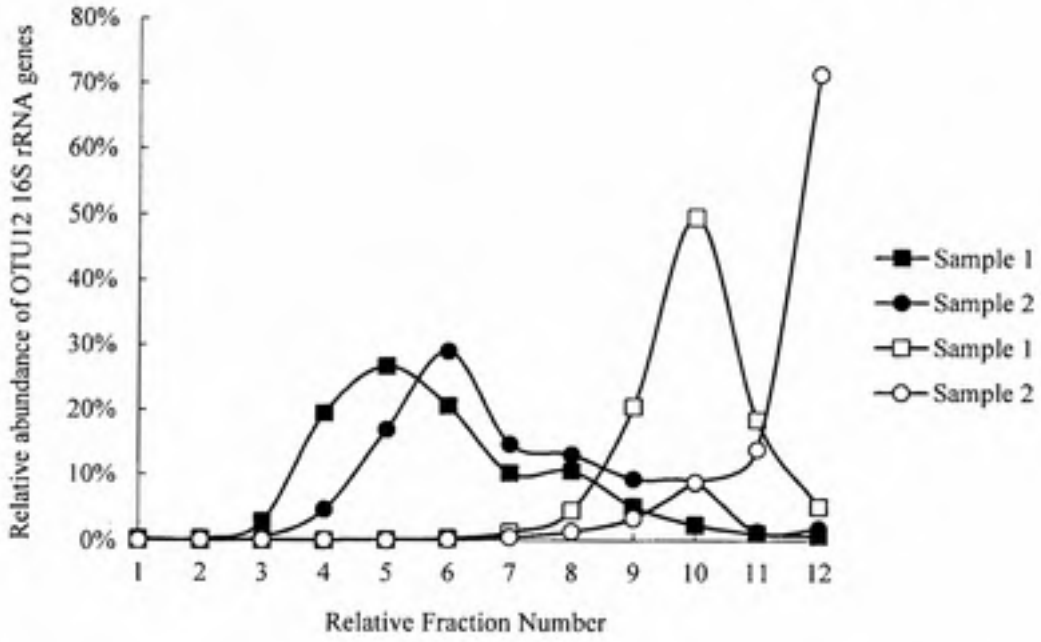


Figure 23: Distribution of OTU-12 16S rRNA genes in previously identified 'heavy' and 'light' relative fractions from incubations with ^{13}C-anthracene (filled symbols), and unlabeled anthracene (open symbols). Points represent the percentage of genes from that OTU present in each fraction as a percentage of the total genes determined for all displayed fractions. Relative fractions 2 through 5 were considered to contain 'heavy' DNA.

d) OTU-19

Members of OTU-19 (representing three out of 43 clones in the libraries derived from heavy DNA) were identified by the RDP-II as being within the genus *Bradyrhizobium* with 100% condifence.

Based on conventional PCR with a linearized plasmid harboring the representative 16S rRNA gene of the OTU, it was determined that the optimum annealing temperature for the PCR primers developed to quantify OTU-19 was 60°C (Figure 24). A qPCR screen of a range of fractions concluded that a significant percentage of 16S rRNA genes associated with OTU-19 (59%) appeared in the heavy fractions of the samples from incubations with ^{13}C-anthracene (Figure 25), whereas only 2% of OTU-19 sequences appeared in the relative heavy fractions of the incubations with unlabeled anthracene.

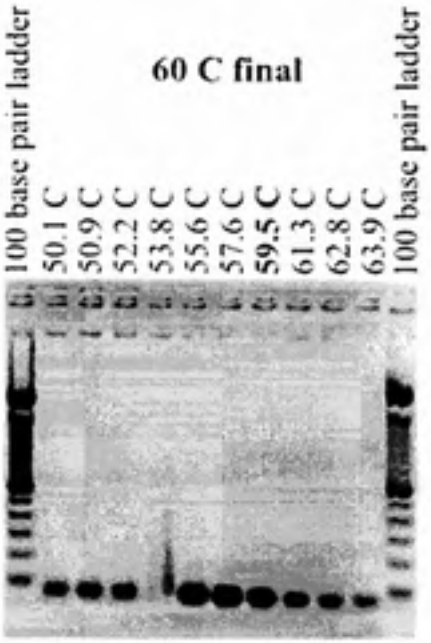


Figure 24: PCR products from OTU-19 digested plasmid and designed qPCR primers at various temperatures.

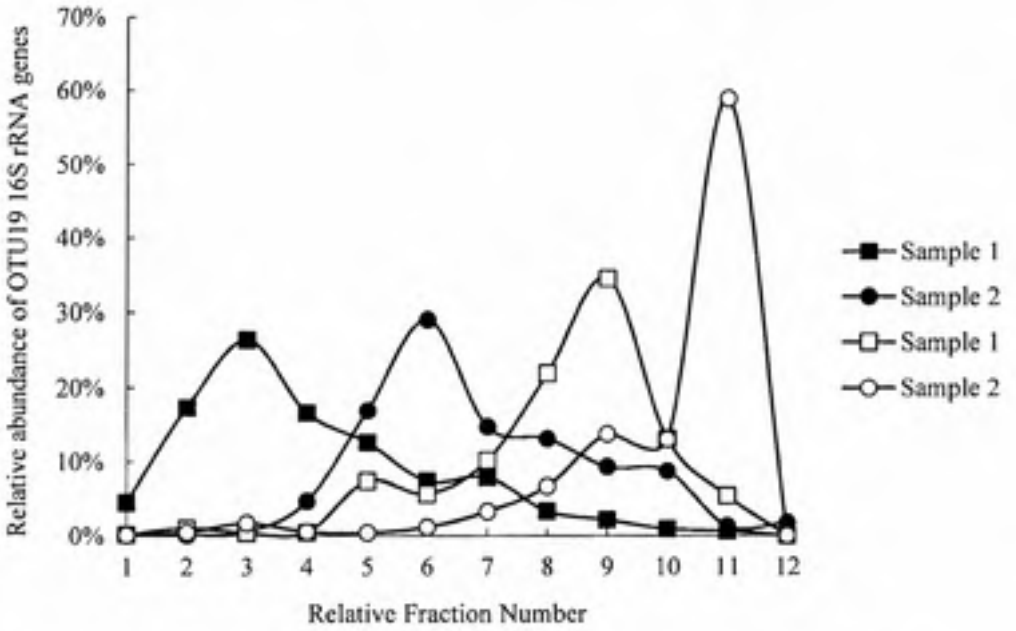


Figure 25: Distribution of OTU-19 16S rRNA genes in previously identified 'heavy' and 'light' relative fractions from incubations with ^{13}C-anthracene (filled symbols), and unlabeled anthracene (open symbols). Points represent the percentage of genes from that OTU present in each fraction as a percentage of the total genes determined for all displayed fractions. Relative fractions 2 through 5 were considered to contain 'heavy' DNA.

e) OTU-6

Based on conventional PCR with a linearized plasmid harboring the representative 16S rRNA gene of the OTU, it was determined that the optimum annealing temperature for the PCR primers developed to quantify OTU-9 was 57.5°C (Figure 26). However, after screening with qPCR at this temperature, the number of 16S rRNA genes in collected fractions were below the quantification limit of the assay, so OTU-6 could not definitively be associated with anthracene degradation. Clones associated with this OTU represented only two of the 43 clones in the libraries constructed from DNA recovered from heavy fractions.

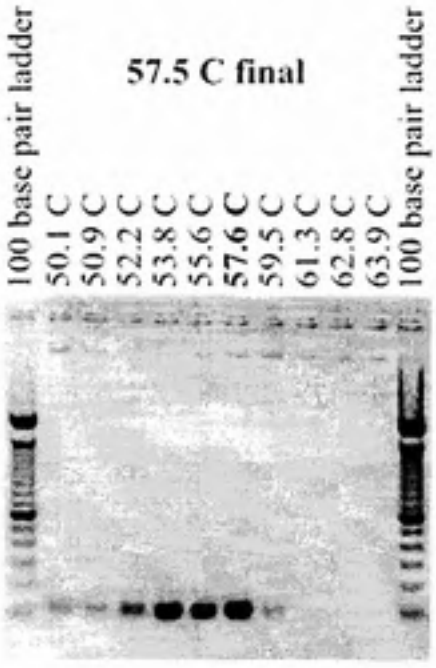


Figure 26: PCR products from OTU-6 digested plasmid and designed qPCR primers at various temperatures.

5. Primer specificity

Each qPCR primer set was tested against the representative sequence from each other quantified OTU to verify the specificity of the primer sets. No products were obtained for any tested reaction with the OTU not targeted by the primer set, with one exception. Primers specific to OTU-19 detected 16S rRNA genes associated with OTU-11 at a level three orders of magnitude lower than the OTU-11 primers (data not shown). This non-specific amplification was not considered significant enough to have influenced quantification of the various OTUs in the SIP experiment.

6. Quantification of SIP-determined OTUs during the course of the SIP incubation

Measuring the change in abundance of bacterial 16S rRNA genes identified in the heavy fractions over the course of the SIP incubations allowed for the identification of bacteria that utilized ^{13}C-enriched anthracene as a carbon and energy source. The results from the time series indicate that 16S rRNA genes associated with all putative anthracene degraders increased over time (Figure 27). However, note that, based on the qPCR analysis of the heavy DNA, PG2 was not considered to have utilized ^{13}C-anthracene. Its presence in the clone libraries of heavy DNA can be explained by the high initial abundance of those organisms in the inoculum and that its 16S rRNA genes did not increase over the course of the SIP incubation (Figure 27). Otherwise, the abundance of quantified genes in each OTU at the end of the SIP incubations was representative of the number of sequences per OTU in the constructed clone library. For example, OTU-11 comprised 20 of 43 clones (47%) and 16S rRNA genes associated with his OTU were

more abundant by almost an order of magnitude than OTU-12 (7 clones) and OTU-19 (3 clones).

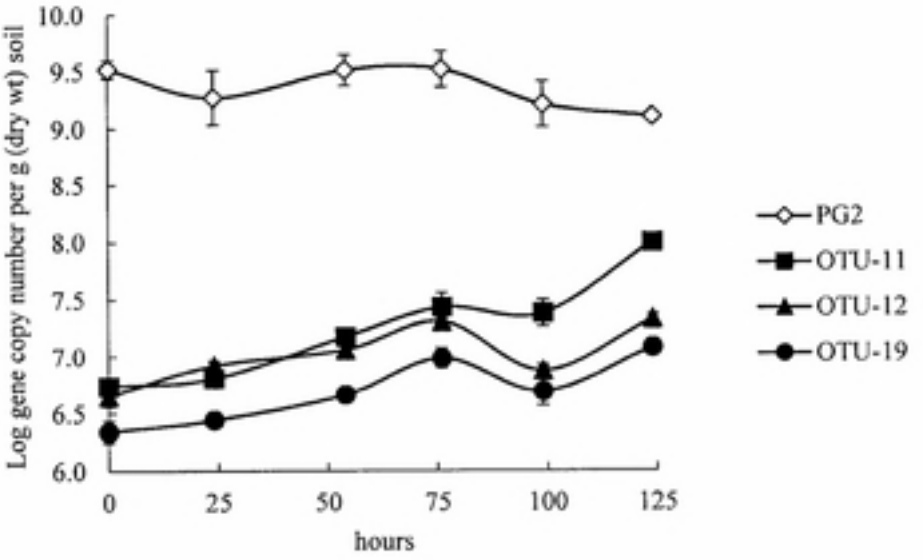


Figure 27: Time course of 16S rRNA gene abundance over time for OTUs from clone libraries of heavy DNA. Filled symbols represent putative anthracene degraders.

V. Discussion

A. "Anthracene Group 1" Presence in Untreated Soil and Bioreactor-Treated Soil

A prior SIP study (Jones et al. 2011a) examining the degradation of anthracene in untreated soil identified 16S rRNA genes from the uncharacterized "anthracene group 1" as the dominant representatives of a clone library constructed from ^{13}C-enriched DNA. In this study, the same soil was analyzed by SIP after treatment in a lab-scale, slurry-phase bioreactor. Three different OTUs of dominant 16S rRNA genes were identified, none of which was closely related to AG1. One OTU was closely related to members of the genus *Altererythrobacter* and two others were similar to bacteria within the order *Rhizobiales*. There are multiple explanations as to why AG1 was not recovered as the dominant anthracene degrader in the bioreactor-treated soil. First, the community of the bioreactor-treated soil has been adapting to biostimulated conditions since December 8, 2008, more than three years before the bioreactor sample was collected for this SIP study. Shifts in bioreactor bacterial communities under different loading conditions have been previously reported (Singleton et al. 2011); that study concluded that not only is the bacterial community of the untreated soil significantly different from the bacterial community in the bioreactor, but also that some bacteria at low relative abundance in the untreated soil were established in the bioreactor. The reverse phenomenon of a high relative abundance organism on the untreated soil decreasing during bioreactor operation was also observed, and was actually the case for AG1 (Singleton et al. 2011). Figure 6 and the prior SIP study (Jones et al. 2011a) provide evidence that AG1 is a viable anthracene degrader in the untreated soil, but this study indicates that AG1 is not likely a central anthracene

degrader in the bioreactor. It is likely that the dominant anthracene-degrading bacteria identified in the bioreactor-treated soil are out-competing AG1, which could be explained by slower growth rates of AG1 organisms than the organisms identified by SIP in this study. The bioreactor environment (continuous agitation, continuous oxygen supply, and relatively high N and P concentrations) is also very different from the field conditions under which the untreated soil was collected. It is possible, therefore, that the anthracene degraders identified in this study are much better adapted to the bioreactor environment than is AG1.

B. PG2 Presence in Bioreactor-Treated Soil

"Pyrene Group 2" (PG2) has been previously studied and identified as a degrader of pyrene (Jones et al. 2008; Jones et al. 2011b; Singleton et al. 2006), phenanthrene (Singleton et al. 2007), fluoranthane and benz[a]anthracene (Jones et al. 2011b) but not naphthalene. In this study, PG2 was identified in the clone library generated from the heavy fractions of the ^{13}C-enriched DNA and represented 6 of 48 clones (13%). However, additional analysis indicated members of PG2 were not likely to have grown on the ^{13}C-anthracene. Members of PG2 could have appeared in the clone library because of its sheer abundance in the sample. The initial concentrations of 16S rRNA genes from PG2 in the bioreactor slurry was at least three orders of magnitude higher than the most abundant SIP-identified anthracene degrader, and two orders of magnitude higher than the other groups of identified anthracene degraders at the end of the experiment. A small percentage of the highly abundant PG2 DNA migrating into the heavy fractions as the leading edge of a very large peak could explain its presence in the clone libraries. This explanation is consistent with the similarity of the distribution of PG2 genes across

ultracentrifuge fractions in samples with ^{13}C-anthracene and from incubations with unlabeled anthracene (Figure 19). Incomplete DNA separation has been previously addressed by Singleton et al. (2005) in a similar SIP experiment and has been long-regarded as a known issue in SIP experiments.

C. Identification of Anthracene-Degrading Bacteria

Three OTUs (OTU-11, OTU-12 and OTU-19) were identified as putative anthracene degraders through the use of SIP. One was closely related to members of the genus *Altererythrobacter* and two others were similar to bacteria within the order *Rhizobiales*.

1. OTU-11

Members of OTU-11 were most closely related to the genus *Altererythrobacter* in the family *Sphingomonadales*. Although this is a relatively newly characterized genus, RDP's newest release contains 147 cultured or uncultured sequences associated with the phylotype. Sequences from this genus have been recovered from several sources including seawater (FM177586, DQ395628, AB302354, EU726272), oil-contaminated soils (JN038296, JN942147, EF157163, EF173348), anthracene-contaminated soil (HM438402, HM438331, HM438326- all from an unpublished study), and one from a pilot-scale bioremediation process of a hydrocarbon-contaminated soil (AM936207). Although Teramoto et al. (2010) proposed an *Altererythrobacter* isolate closely related to *Altererythrobacter epoxidivorans* as a potential PAH-degrader in nutrient-rich tropical environments, it would appear that *Altererythrobacter* species are adapted to many environments, including PAH-contaminated sites, thus adding to its sustainability and viability in the environment.

2. OTU-12 and OTU-19

16S rRNA genes sequenced from members of OTU-12 and OTU-19 were both associated with the order *Rhizobiales*, with OTU-19 additionally associated with the genus *Bradyrhizobium*. Many members of the genus *Rhizobiales* are associated with nitrogen fixation and some have been linked to degradation of anthracene under methanogenic conditions (Zhang et al. 2011) and in a two-liquid phase bioreactor system (Lafortune et al. 2009). Whether there is a possible link of nitrogen fixation to be the presence of organisms from these two OTUs in the bioreactor is open to question. Hanson et al. (2012) recently published a paper in which evidence was presented that nitrogen fixation by *Polaromonas napthalenivorans* strain CJ2, in coal tar-contaminated sediments, may play a role in the biodegradation of naphthalene.

D. Implications for Bioremediation

This study adds to the body of knowledge on bioremediation of PAH-contaminated soil by demonstrating that the natural microbial community in aged, PAH-contaminated soil is not necessarily the community that degrades PAHs in an engineered system. In other words, knowledge of organisms capable of degrading a specific contaminant in an untreated soil is not necessarily predictive under a bioremediation scenario. MacNaughton et al. (1999) noted a substantial change in the diversity of the dominant bacterial community in response to an experimental oil spill. Vinas et al. (2005) noted shifts in bacterial communities not only if treatment was performed but also in response to the type of nutrient used in the treatment process. Lors et al. (2012) however, recently compared a field biotreatement and a lab scale treatment of the same soil samples over 182 days, and discovered the temporal appearance and disappearance of

microbial strains were nearly identical in both samples. Knowing which bacteria are likely to be the dominant degraders of a contaminant under a specific bioremediation strategy is important in order to predict the potential success of the treatment as well as to tailor the conditions of the engineered bioremediation processes for the greatest removal of those contaminants (Martin et al. 2012). Consistent with principles of microbial ecology, it is not surprising that environmental conditions exert a strong influence on members of a microbial community. However, it is somewhat surprising that organisms highly abundant in untreated soil would not have a competitive advantage over organisms initially present in low abundance.

The findings in this study are consistent with the concept of functional redundancy in engineered biological treatment systems that rely on microbial communities to perform desired functions (Curtis and Sloan 2006; Korotkevych et al. 2011; Pilloni et al. 2011). Functional redundancy implies that a microbial community can have multiple members that are capable of carrying out a particular function (in this case, growth on anthracene), such that the function is manifested regardless of which member(s) of the community are selected under a given set of conditions.

E. Methodological Observations

Before carrying out the SIP experiment, preliminary experiments were performed to identify conditions that would limit the variability of the pH during incubations. In addition, possible outcomes of a decreased disappearance rate in the SIP experiment as compared to the preliminary experiment were examined.

The concentration of ammonium nitrate was observed to influence the pH of incubations. Similar pH issues have been previously documented by others (Erickson 1993; Jacques et al. 2007; Kastner et al. 1998; Singleton et al. 2011). A prior study on the effect of pH on PAH degradation concluded that degradation was enhanced at a pH of 7.0 compared to a pH of 5.2 (Lu et al. 2011). Because pH is a master environmental variable, a lower or changing pH could substantially impact the PAH degradation rates in addition to shifting the populations in the microbial community. In this study, although the overall number of bacterial 16S rRNA genes in the tested soil did not decrease significantly when the pH dropped to 4.8 during an incubation with a phosphate buffer containing 5.0 mM ammonium nitrate, it is unknown if the degradation rates of PAHs were impacted or how the community varied from incubations that resulted in a higher terminal pH. Ultimately, to reduce the impact of a lower pH in the SIP incubations, the ammonium nitrate concentration in the utilized phosphate buffer was adjusted to 2.5 mM. This concentration provided minimal pH changes while increasing the rates of ^{14}C-anthracene mineralization and anthracene disappearance relative to incubations with 5 mM ammonium nitrate. The fact that reducing the ammonium nitrate concentration led to a more stable pH is consistent with nitrification as the mechanism by which pH decreased in incubations with 5 mM ammonium nitrate. Ammonium oxidation is known to consume alkalinity (produce acid).

During the SIP experiment, anthracene disappearance did not follow the same pattern as during preliminary tests. However, the preliminary tests were used to identify the duration of the SIP incubations. If this observation was not merely due to technical issues during the extraction and quantification of anthracene, a decreased disappearance

rate could mean less incorporation of ^{13}C-enriched anthracene. Such a scenario would lead to less incorporation of labeled carbon and possibly less separation of heavy and light DNA. This may explain why two distinct DNA bands were not observed when the SIP ultracentrifugation tubes were examined visually. The untreated soil showed a greater rate of disappearance than the bioreactor-treated soil, but there was no evidence of heavy DNA in those samples. Fractionation of the bioreactor-treated soil tubes resulted in distinct heavy and light fractions of DNA. It is possible that anthracene concentration was not accurately measured by HPLC due to uneven distribution of anthracene in the slurry. It is also possible that not enough time had passed for the untreated soil to utilize the ^{13}C-anthracene. A prior SIP study (Jones et al. 2011a) utilizing untreated soil and [U-^{13}C]anthracene produced heavy and light fractions after a time period of 20 days, whereas the majority of anthracene in the this experiment was removed within three days.

F. Recommendations

Following are recommendations for additional studies that could extend the findings of this study.

1. Quantifying the three identified anthracene degraders in the untreated soil would allow for it to be determined if AG1 is out-competing them in incubations with untreated soil but not in the bioreactor.
2. Regarding OTU-19, an additional study could be performed to track nitrogen-fixation activity. Should a representative of OTU-19 be isolated, examining its genome for nitrogen fixation genes may provide insight into how this microorganism continues to stay relevant in the bioreactor community despite being at low levels.

3. A study could be conducted to evaluate how inoculation of untreated soil with bioreactor slurry might affect the dominant anthracene degraders.
4. Isolating either AG1 or a representative of the OTUs responsible for anthracene degradation in the bioreactor would allow for extensive investigation of the physiology of anthracene degradation by environmentally relevant bacteria.

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